The Tohoku Journal of Experimental Medicine Vol. 64, Nos. 3-4, 1956

Significance of Intravenous Double Sugar Loading Test. Criticism of the Staub Effect

By

Yoshio Goto
(後藤 由夫)

From the Medical Clinic of Prof. T. Kurokawa,
Tohoku University, Sendai

(Received for publication, September 22, 1955)

The double sugar loading test has been generally accepted as the routine examination to detect and classify the diabetes mellitus, since the Staub effect (Hamman-Hirschman phenomenon) was understood as the result of the overproduction of insulin from the pancreas. The mechanism of the occurrence of this phenomenon has not been made entirely clear. In the previous paper, it was demonstrated that the double sugar loading curve could be explained as a sole summation of the two single sugar loading curves.[1] The reason why the second curve is lower than the first is that the second hyperglycemia is diminished by the hypoglycemia due to the prior ingestion of glucose. Therefore, we cannot expect any special significance and value over and above that of the single loading test for the double loading test. In this paper, the Staub effect was re-examined by the intravenous glucose loading test, making judgment by a comparison of the degree of hyperglycemia and the form of the glucose disappearance curves.

Experimental

Method

Diabetic and non-diabetic patients were used in this experiment. The same care was taken as described in the previous paper. Two kinds of sugar loading test were performed, the single loading test and the double loading test. In each case, 15 g. of glucose as a 50% solution was administered intravenously once or twice.

Single glucose loading test. After overnight fasting, an initial blood specimen was taken and the glucose solution was injected within two minutes. Blood specimens were collected from the ear lobe for the blood sugar estimation at three minutes after the performance of the glucose injection and subsequently every ten minutes for three hours.

Double glucose loading test. Within next two days, double loading test

was performed on the same individual. After overnight fasting, 15 g. of glucose were administered twice with an interval of seventy minutes and the blood specimens were taken at every ten minutes for three hours.

Blood sugar estimations were made in non-duplicate, according to the micro-method of Fujita and Iwatake.[2]

The arithmetical construction of the presumed double glucose loading curve was made from the measured single glucose loading curve in the same way in the previous report as shown in Fig. 1. The magnitudes of hyperglycemia were calculated by subtracting the fasting blood sugar value from the values measured at 3 minutes, 13 minutes, 23 minutes and so on. These calculated values were added to the blood sugar values measured at 73 minutes, 83 minutes, 93 minutes . . . 183 minutes respectively, as shown in Fig. 1. In this way, the theoretical double loading curve was obtained. This theoretical curve was compared with the measured double loading curve.

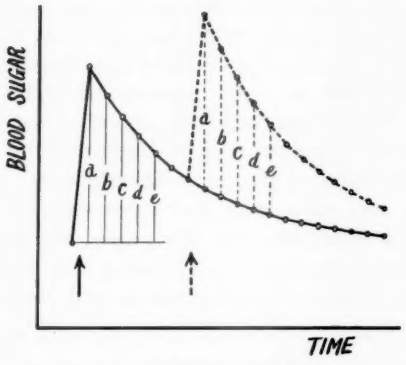


Fig. 1. Drawing of presumed intravenous double sugar loading curve. The continuous line represents the measured single sugar loading curve; the broken line represents the presumed second curve. The solid arrow indicates the administration of glucose and the broken arrow indicates the assumed administration of glucose.

In order to keep the fasting blood sugar in a constant level (homeostatic blood sugar level or " Regulationspunkt " named by Kuzuya[3]), 5 units of insulin was injected five or six hours before the test (Table I, Cases 1, 3, 8, 9). In a few cases, the presumed second curves were corrected with the measured spontaneous depression curves of fasting blood sugar, as mentioned in the previous paper (Cases 5, 6, 7). In Cases 18 and 19, the glucose solutions were injected at an interval of thirty minutes and in Case 17, 20 g. of glucose were injected with a eighty minutes interval.

Results

The experimental results are summarized in Table I and some of them are illustrated (Figs. 2, 3). The measured blood sugar values correspond to the presumed blood sugar values. The forms of these two glucose disappearance curves, measured and presumed ones, show great similarities either in diabetics or in non-diabetics.

To judge the results objectively, a further scrutiny was attempted.

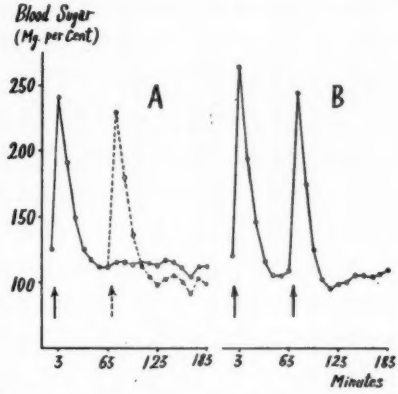


Fig. 2. A 29 year-old male, non-diabetic (Case 15). A, the continuous line represents the measured single loading curve; the broken line represents presupposed second curve. B, measured double loading curve. The arrows indicate the injection of glucose solution.

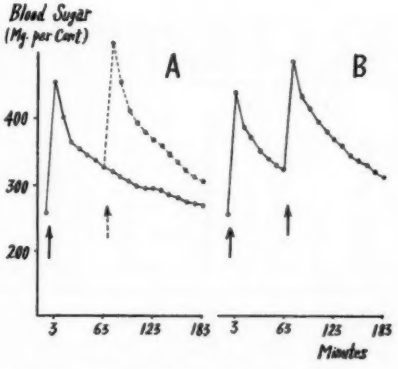


Fig. 3. A 64 year-old female, diabetic (Case 3). Same as in Fig. 2.

TABLE I

Case No.	Age, Sex	Disease	Loading test	Blood Sugar (Mg. per cent)*																		
				Fasting	3′	13′	23′	33′	43′	53′	63′	73′	83′	93′	103′	113′	123′	133′	143′	153′	163′	173′	183′
1	47 M	Diabetes mellitus	Single	139	288	231	217	208	196	187	184	180	175	172	170	165	160	156	154	151	149	147	144
												(329)	(267)	(250)	(239)	(222)	(208)	(201)	(195)	(187)	(182)	(178)	(170)
			Double	153	298	247	235	224	215	207	201	335	281	262	257	241	235	229	217	215	207	205	196
2	38 M	Diabetes mellitus	Single	120	236	200	182	168	154	142	135	127	114	107	102	102	104	100	96	97	96	97	102
												(243)	(194)	(169)	(150)	(136)	(126)	(115)	(103)	(91)	(83)	(79)	(84)
			Double	118	237	200	184	172	160	149	135	246	196	177	158	142	132	118	106	97	94	90	87
3	64 F	Diabetes mellitus	Single	259	455	402	364	354	344	334	326	320	312	306	298	294	294	292	284	280	274	272	270
												(516)	(455)	(411)	(393)	(379)	(369)	(359)	(345)	(333)	(321)	(311)	(305)
			Double	259	440	388	372	350	340	330	322	486	434	416	396	380	368	358	344	336	330	320	312
4	38 M	Diabetes mellitus	Single	111	241	189	163	142	127	112	104	96	96	96	94	94	94	97	96	97	96	96	94
												(226)	(174)	(148)	(125)	(110)	(95)	(90)	(81)	(82)	(81)	(79)	(77)
			Double	120	247	194	168	154	135	123	112	236	180	153	137	121	107	99	92	90	87	85	90
5	41 F	Diabetes mellitus	Single	158	290	257	233	217	200	186	177	165	158	149	142	140	135	133	132	130	130	121	123
												(297)	(258)	(226)	(204)	(187)	(169)	(159)	(147)	(139)	(130)	(116)	(117)
			Double	149	283	261	236	217	203	187	180	306	280	247	227	208	194	184	174	165	154	153	153
6	65 F	Diabetes mellitus	Single	139	292	235	214	194	177	165	154	144	135	133	132	128	125	121	120	118	116	116	111
												(297)	(231)	(210)	(190)	(171)	(159)	(146)	(136)	(126)	(123)	(123)	(115)
			Double	139	287	236	214	200	184	167	158	306	247	219	205	187	174	150	140	134	130	120	117
7	53 F	Diabetes mellitus	Single	148	316	254	229	214	198	184	172	163	151	144	137	130	125	121	120	116	112	107	109
												(329)	(257)	(231)	(213)	(191)	(175)	(163)	(153)	(142)	(133)	(124)	(124)
			Double	156	322	261	238	222	205	193	182	350	278	248	231	214	198	189	177	164	154	146	139
8	62 F	Diabetes mellitus	Single	220	364	318	298	286	274	262	254	246	240	236	232	229	226	222	218	213	208	201	195
												(390)	(338)	(314)	(298)	(283)	(268)	(256)	(244)	(233)	(224)	(213)	(204)
			Double	218	356	330	302	288	270	266	256	392	354	326	298	292	280	266	260	250	246	236	228
9	62 M	Diabetes mellitus	Single	182	334	266	252	238	227	219	212	205	198	194	191	187	186	182	180	182	182	180	180
												(357)	(282)	(264)	(247)	(232)	(223)	(212)	(203)	(198)	(194)	(193)	(187)
			Double	212	364	302	287	276	267	259	252	388	323	304	294	280	269	262	254	248	241	236	229

10	54 F	Portal cirrhosis	Single	92	219	177	147	132	114	100	92	87	85	85	83	83	81	80	81	81	81	80	85
												(214)	(170)	(140)	(123)	(103)	(89)	(80)	(76)	(74)	(74)	(71)	(76)
			Double	92	222	174	154	132	120	104	100	218	179	146	125	109	97	96	92	90	88	92	90
11	45 F	Serum hepatitis	Single	114	261	223	191	165	142	120	109	104	101	99	97	94	94	94	94	96	94	96	96
												(251)	(210)	(176)	(148)	(122)	(100)	(89)	(84)	(87)	(83)	(79)	(76)
			Double	114	252	220	184	161	139	120	109	245	201	169	144	121	107	94	87	78	80	81	85
12	30 M	Chronic colitis	Single	97	250	215	186	163	147	132	118	99	92	87	80	85	85	87	88	92	88	88	88
												(252)	(210)	(176)	(146)	(135)	(120)	(108)	(90)	(87)	(78)	(71)	(76)
			Double	99	259	219	189	168	144	125	104	248	201	165	146	132	116	104	96	85	73	69	71
13	56 F	Chronic Pancreatitis	Single	107	282	222	191	161	139	111	96	83	81	85	88	94	97	97	97	97	97	97	97
												(258)	(196)	(169)	(142)	(126)	(101)	(86)	(73)	(71)	(75)	(78)	(84)
			Double	107	287	220	191	151	120	104	88	261	193	153	114	94	81	83	85	88	93	97	97
14	30 M	Gastritis	Single	99	259	196	161	140	121	109	99	97	97	96	94	87	88	90	92	94	97	97	104
												(237)	(149)	(156)	(131)	(109)	(98)	(90)	(90)	(92)	(92)	(88)	(92)
			Double	103	261	200	158	144	121	111	111	242	186	146	121	107	106	102	100	100	107	112	111
15	29 M	Lymphadenitis tuberculosa	Single	127	241	191	149	125	118	112	112	116	116	114	116	114	113	118	116	112	104	112	112
												(230)	(180)	(136)	(114)	(105)	(98)	(103)	(105)	(101)	(91)	(103)	(99)
			Double	121	264	194	146	116	106	106	109	247	174	125	102	94	99	100	106	106	104	106	109
16	53 M	Splanchnoptosis	Single	106	224	175	135	111	94	87	81	81	83	83	85	85	85	87	87	87	87	89	89
												(199)	(152)	(112)	(90)	(73)	(66)	(62)	(64)	(64)	(66)	(68)	(70)
			Double	102	233	177	132	111	96	85	83	209	160	118	100	92	85	76	73	80	81	81	83
17	31 F	Gastritis	Single	106	354	287	245	214	191	170	151	133	120	106	97	87	83	81	78	78	83	88	92
													(368)	(287)	(236)	(195)	(168)	(145)	(123)	(105)	(97)	(88)	(83)
			Double	106	367	285	241	208	184	160	144	127	374	285	238	198	160	128	102	88	80	74	76
18	56 M	Esophageal cancer	Single	107	203	163	146	137	127	116	109	104	100	97	97	97	97	96	97	99			
								(233)	(183)	(155)	(139)	(124)	(109)	(99)	(94)	(90)	(87)	(86)	(87)	(89)			
			Double	107	193	161	144	236	189	172	156	142	133	125	116	107	99	92	87	85			
19	16 F	Acute pancreatitis	Single	106	233	184	158	142	128	116	107	102	99	97	97	96	94	97	99	99	99	96	100
								(269)	(206)	(168)	(143)	(124)	(109)	(99)	(93)	(89)	(85)	(88)	(89)	(87)	(90)	(89)	(93)
			Double	107	231	182	156	259	207	168	143	125	106	95	90	83	81	83	85	90	96	96	99
20	44 M	Diabetes mellitus	Double	172	280	259	242	226	215	200	191	299	257	233	226	214	194	190	187	177	172	161	151

* The values in parentheses represent the calculated values.

It was proved by Nonobe[4] that the equation of one-dimensional diffusion could be applied to the glucose disappearance curve. The equation is

$$y(t) = a\ e^{-bt} + C$$

where e is the base of natural logarithm, and a, b, C are constants. We calculated these values for measured double loading curves and for the presumed double loading curves (Table II, E and B). According to Matuda,[5] differences less than 0.05 in e^{-b} and differences less than 10 in C can be regarded as technical errors.

Generally speaking, both values show a fairly good coincidence. The cases, in which differences in these two values of e^{-b} are greater than 0.05, are the Cases 14 and 18; and the cases, in which the differences in these two values of C are greater than 10, are the Cases 2, 5, 9, 14, 15, 17 and 18. However, in Case 9, the first glucose disappearance curve of double loading curve is so different from that of the single loading curve, that it is not proper to compare the measured second curve with the presumed second curve, because in this case the sugar assimilating function of the body seems to change according to the test days. Then we used another method to construct the presumed second curve from the first disappearance curve of the double loading curve by calculation. Let us denote the fasting blood sugar level by F. Then the magnitude of blood sugar due to the glucose injection can be represented as

$$a\ e^{-bt} + C - F$$

The second glucose disappearance curve due to the second glucose injection at the time n, can be represented as

$$\begin{aligned} y(t) &= a\ e^{-bt} + C + a\ e^{-b(t+n)} + C - F \\ &= a(1+e^{-bn})e^{-bt} + 2C - F \end{aligned}$$

Thus we can calculated the values of a, e^{-b}, C of the assumed second glucose disappearance curve from the values of a, e^{-b}, C of the first curve of the measured double glucose loading curve. The presumed second curve obtained by such a way, corresponds with the measured second curve as shown in Fig. 4. Of course, this calculation is based upon a premise that the carbohydrate assimilating power of the body does not change from time to time, but it is rather constant. This method of comparison of two curves was applied to all cases (Table II).

Discussion

The Staub effect is usually demonstrated by the oral double sugar administration, as reviewed briefly in the previous paper.[1] However, subsequent investigators attempted to examine this effect with the in-

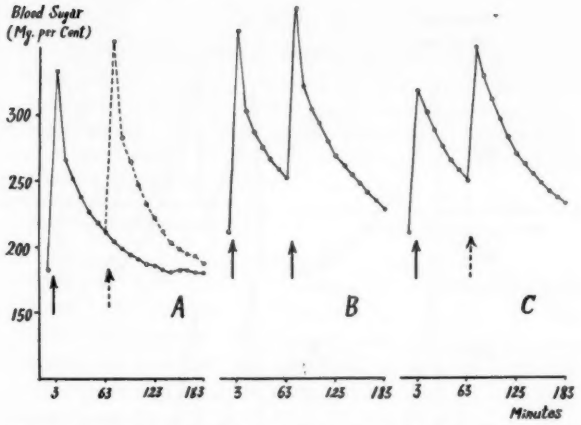


Fig. 4. A 62 year-old male, diabetic (Case 9). A, measured single sugar loading curve and presumed second curve. B, measured double loading curve. C, presumed double loading curve obtained from the first curve of measured double loading curve (B) by calculation. (See text). B curve is different from the A curve, but corresponds with C cure.

travenous sugar administration, because of the instability of the resorption of sugar from the intestine.

Terada[6] performed the intravenous double glucose loading test in the rabbits with the damaged liver and concluded that the liver plays a great role in the occurrence of the Staub effect. On the other hand, Soskin[7] postulated the indispensability of hypophysis in the occurrence of the Staub effect. The intravenous double glucose loading test was examined by Ito[8] in diabetics, patients with hepatic or pancreatic diseases and healthy adults. He found that, when 4 g. of glucose were injected at an interval of 30 minutes, the Staub effect almost appeared in healthy adults, but when 10 g. of glucose were injected at the same interval, this effect sometimes did not appear. He postulated further that the intravenous single loading test was more suitable than the double loading test for making an inference of the sugar assimilating function in the body. Pletscher and his co-workers[9] postulated from the results of th double glucose loading test in normal, depancreatized and depancreatized-insulinized dogs, that the Staub effect would appear as a result of extra-insulin.

These authors judge the Staub effect only by the comparison of the height of the peaks in the first and the second disappearance curves. The present experimental results clearly show that the relations of the two-peaks, the first peak higher than the second or the first peak lower than

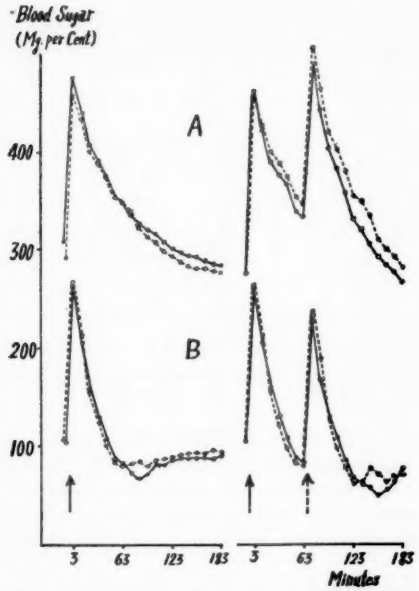


Fig. 5. Intravenous single sugar loading curves of the same individual obtained on the different test days and the presumed double loading curves constructed by calculation from them. A, a 67 year-old female, diabetic, and B, a 53 year-old female, non-diabetic.

the second, are quite the same between the measured double loading curves and the theoretical double loading curves. Therefore, as far as the comparison of the height of the two peaks is concerned, the Staub effect is of no special significance.

Kosaka[10] re-examined the Staub effect in normal and depancreatized diabetic dogs with intravenous loading test. He applied the equation of one-dimensional diffusion[4] to the first and the second glucose disappearance curves and compared the values of a, e^{-b} and C of the first curves with those of the second curves in order to judge the occurrence of the Staub effect. He found that the Staub effect could be recognized in the cases of the normal dogs in which two doses of glucose were injected at 120-minute interval, and in other cases in which sugar doses were injected at a shorter interval than 120 minutes, the Staub effect could not be demonstrated.

If we want to examine the change in the assimilating function of the body by the double sugar loading test, that is, to discuss the Stub effect, we must judge it from a comparison of the measured and calculated second

TABLE II

Fomulas of Glucose Disappearance Curves

A, measured single loading curve, B, presumed second disappearance curve obtained by calculation, C, first disappearance curve of measured double sugar loading curve, D, presumed second curve obtained from the first curve of the measured double loading curve by calculation (see text), E, measured second disappearance curve.

Case No.	Single Loading Curve A	B	Double Loading Curve C	D	E
1	$106(0.88)^t+138$	$154(0.88)^t+133$	$117(0.88)^t+144$	$164(0.88)^t+135$	$156(0.89)^t+142$
2	$143(0.85)^t+78$	$184(0.86)^t+36$	$133(0.87)^t+85$	$183(0.87)^t+52$	$163(0.87)^t+55$
3	$159(0.89)^t+261$	$228(0.89)^t+257$	$154(0.89)^t+253$	$220(0.89)^t+247$	$203(0.88)^t+257$
4	$145(0.77)^t+76$	$156(0.80)^t+51$	$140(0.79)^t+75$	$166(0.79)^t+38$	$153(0.80)^t+58$
5	$160(0.82)^t+126$	$202(0.83)^t+91$	$160(0.82)^t+130$	$199(0.82)^t+111$	$196(0.83)^t+114$
6	$156(0.81)^t+108$	$162(0.84)^t+95$	$147(0.81)^t+117$	$180(0.81)^t+105$	$176(0.83)^t+101$
7	$187(0.87)^t+92$	$218(0.88)^t+65$	$189(0.88)^t+94$	$266(0.88)^t+32$	$232(0.89)^t+70$
8	$132(0.84)^t+208$	$178(0.85)^t+188$	$157(0.82)^t+202$	$199(0.82)^t+186$	$182(0.83)^t+197$
9	$118(0.85)^t+166$	$142(0.85)^t+161$	$108(0.85)^t+211$	$142(0.85)^t+210$	$142(0.85)^t+203$
10	$154(0.73)^t+65$	$162(0.76)^t+49$	$133(0.75)^t+75$	$150(0.75)^t+58$	$163(0.77)^t+55$
11	$199(0.81)^t+62$	$221(0.83)^t+32$	$203(0.80)^t+58$	$245(0.80)^t+2$	$201(0.81)^t+38$
12	$184(0.82)^t+65$	$201(0.82)^t+42$	$198(0.81)^t+59$	$243(0.81)^t+19$	$195(0.80)^t+45$
13	$208(0.72)^t+72$	$207(0.74)^t+42$	$227(0.69)^t+64$	$243(0.69)^t+21$	$216(0.70)^t+42$
14	$166(0.74)^t+72$	$183(0.75)^t+57$	$158(0.64)^t+99$	$164(0.64)^t+94$	$159(0.65)^t+83$
15	$165(0.63)^t+88$	$166(0.63)^t+75$	$186(0.62)^t+80$	$192(0.62)^t+39$	$195(0.60)^t+57$
16	$170(0.62)^t+70$	$157(0.62)^t+55$	$167(0.65)^t+70$	$175(0.65)^t+48$	$147(0.66)^t+62$
17	$269(0.82)^t+64$	$311(0.80)^t+38$	$272(0.81)^t+65$	$334(0.81)^t+24$	$321(0.80)^t+27$
18	$104(0.78)^t+84$	$152(0.78)^t+65$			$159(0.87)^t+50$
19	$137(0.75)^t+80$	$186(0.74)^t+67$			$193(0.76)^t+60$
20			$149(0.85)^t+131$	$196(0.85)^t+90$	$182(0.85)^t+103$

curves. If we find great differences in measured and calculated second curves, we can admit the change in the assimilating function of the body caused by the repeated injections of glucose. In many cases of non-diabetics, the measured second curves are likely to exist below the assumed second curves. On the contrary, in many cases of diabetics the measured second curves are likely to exist above the presumed second curves. These differences seem to be suitable for the supportance of existence of the Staub effect. In normal adults, the assimilating power of the body is aggravated by the repeated sugar administration and on the contrary in diabetic patients, this power is diminished by the repeated administration of the sugar. However, these differences are very slight and sometimes slighter than the differences between two assumed second curves which

are obtained from two single loading curves obtained in the same test subject (Fig. 5).

I have no mind to explode the idea of the Staub effect fundamentally, but I think this effect is not so remarkable and demonstrable as that was thought generally and therefore it is of no special meaning for the clinical application.

From the present experimental results, it can be concluded that the intravenous double sugar loading test is no more adequate test than the intravenous single sugar loading test for the clinical application and has no special significance.

Summary

Intravenous single glucose loading test and intravenous double glucose loading test were performed in the same individuals, diabetics and non-diabetics. The presumed double loading curve was constructed from the single loading curve by calculation, and the actual double loading curve was compared with the presumed double loading curve. The results show that the calculated curves closely correspond to the measured loading curves. From these results, the intravenous double sugar loading test is of no special significance.

References

1) Goto, Metabolism, 1955, **4**, 323.
2) Fujita & Iwatake, Biochem. Z., 1931, **242**, 43.
3) Kuzuya, Tokyo Igakukai Z., 1941, **55**, 60.
4) Nonobe, Sogo Igaku, 1948, **5**, 830.
5) Matuda, Tohoku Igaku Z., 1954, **49**, 693.
6) Terada, Tokyo Ijishinshi, 1939, **63**, 1125.
7) Soskin, Mirsky, Zimmerman & Heller, Amer. J. Physiol., 1936, **116**, 148.
8) Ito, Tohoku Igaku Z., 1948, **38**, 151.
9) Pletscher, Staub, Hunzinger, & Hess, Helv. physiol. Acta, 1950, **8**, 306.
10) Kosaka & Mizuno, Saishin Igaku, 1953, **8**, 991.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Liver Biopsy Findings of Malnourished Infants with Severe Rickets Studies on the Nutrition of Children at Hirosaki Area

(36th Report)

By

Yuji Sato

(佐藤 雄治)

From the Department of Pediatrics of Prof. Ts. Arakawa and from the Department of Pathology of Prof. K. Sato, School of Medicine, Hirosaki University, Hirosaki

(Received for publication, September 22, 1955)

In my preceding paper[1] I reported liver biopsy findings of children who showed marked signs of ariboflavinosis and/or pellagrous skin lesions as part manifestations of nutritional dystrophy and who were not affected by any other severe illness.

In the present investigation liver biopsy was carried out on such infants, in whom clinical symptoms of rickets were conspicuous as compared with such signs as arisen from other nutritional deficiencies.

Materials and Method

In all the 22 cases biopsied, the diagnosis[2] of florid rickets was established both by clinical symptoms (rickety rosary, Harrison's sulcus and double joint of the wrist) and by radiological findings (cupping in the distal end of ulna or irregular and rough appearance of end of diaphysis of ulna and/or radius).

Angular stomatitis, cheilosis, swelling and/or atrophy of lingual papillae, hyperpigmentation of the skin and palpable or enlarged liver were recorded as part manifestations of nutritional dystrophy.[3][4][5]

The method of needle biopsy of the liver and the description of histological findings of the biopsied specimens were the same as in my preceding report.[1]

Results and Comment

The results obtained are given in Tables I A and I B (Cf. Tables I A and I B). Fatty infiltration was found in 12 cases (Cases 1–4, 6, 8, 11–14, 18 and 21). Plant cell-like appearance of hepatic cells was seen in 12 cases (Cases 1, 7, 9, 10, 13, 15–17 and 19–22). Cloudy swelling

TABLE I (A)

Liver Biopsy Findings of Malnourished Infants with Severe Rickets

Groups	No. of case (No. of biopsy)	Name, sex and age (y.m.)	Method of feeding for the first 12 months of life		Signs of nutritional dystrophy				Complications
			Mostly	Partly	Hyperpigmentation	Aribofla-vinosis	Edema	Liver	
Group I	1 (227)	K.S. ♀ (0.4.)	Rice flour	M.M.*	++	−	+	2.5	
	2 (157)	M.J. ♂ (0.6.)	Rice flour + S.C.M.†	M.M.	−	−	−	6	Acute bronchitis
	3 (179)	S.S. ♂ (0.8.)	Rice flour		++	+	−	3.5	
	4 (176)	T.A. ♂ (0.10.)	M.M.	Rice flour	−	−	−	3.5	Prematurity
	5 (160)	S.M. ♂ (1.10.)	M.M.		−	−	−	5	
Group II	6 (263)	N.B. ♂ (1.2.)	Rice flour		++	+	−	3.5	
	7 (214)	Y.Sh. ♀ (1.0.)	Rice flour	S.C.M.	++	++	−	4	
	8 (255)	S.E. ♀ (1.3.)	Rice flour		−	++	+	5	
	9 (222)	N.S. ♀ (1.5.)	M.M.		−	−	−	4	
	10 (272)	K.H. ♀ (1.5.)	Powdered milk	Rice flour	−	−	−	6	
	11 (152)	S.H. ♀ (1.7.)	Powdered milk	Rice flour	−	++	−	7	
	12 (171)	S.Y. ♂ (1.3.)	Powdered milk		++	+	−	3.5	
	13 (319)	A.H. ♂ (1.3.)	M.M.	Powdered milk	−	+	−	3.5	
	14 (175)	I.S. ♀ (1.8.)	Rice flour		±	++	−	3.5	
	15 (279)	S.H. ♂ (1.10.)	M.M.		−	+	−	5	
Group III	16 (249)	O.K. ♂ (3.3.)	Powdered milk	Rice flour	−	++	−	3.5	
	17 (271)	F.K. ♂ (2.8.)	Rice flour	S.C.M.	−	−	−	2	Pertussis
	18 (252)	N.F. ♀ (2.11.)	Rice flour		−	++	−	4	Tetany
	19 (223)	Y.K. ♂ (2.3.)	Rice flour	M.M.	+	++	+	3.5	
	20 (224)	S.Y. ♀ (2.6.)	M.M.	Powdered milk	−	−	−	6	
	21 (170)	M.M. ♂ (2.10.)	Rice flour	S.C.M.	+	+	−	4	
	22 (236)	I.T. ♂ (3.2.)	Rice flour	M.M.	+	++	+	4	

TABLE I (B)

Liver Biopsy Findings of Malnourished Infants with Severe Rickets

Groups	No. of case (No. of biopsy)	Change in hepatic cells					Change in nuclei of hepatic cells	Hypertrophy of kupffer's cells	Cell infiltration inthe portal tracts	Diffuse fibrosis
		Fatty		Clovdy swelling	Plant cell-like appearance	Zonal necrosis				
		Crops	Droplets							
Group I	1 (227)	++	++	+	+	+	+	+	+	
	2 (157)	+	++						+	
	3 (179)	+++	++	+			+		+	++
	4 (176)		+			+	+		+	
	5 (160)			++						
Group II	6 (263)		++	+					+	
	7 (214)			+	++				+	
	8 (255)		+++	++		+				++
	9 (222)				++		+			
	10 (272)				++					
	11 (152)	+	++	+++					+	
	12 (171)	+		++						±
	13 (319)	+			++		+			+
	14 (179)		+	+++						++
	15 (279)			+	+++		+	+		+
Group III	16 (249)			++	++					++
	17 (271)			+++	+					
	18 (252)	+++	+++							
	19 (232)				+++					
	20 (224)			+	+++		+			
	21 (170)	+		+	++					
	22 (236)				+++					

occurred in 14 cases (Cases 1, 3, 5, 6–8, 11, 12, 14–17, 20 and 21), zonal necrosis in 3 cases (Cases 1, 4 and 8), and diffuse fibrosis in 8 cases (Cases 3, 8, 12–16 and 22).

From the results shown above, it will be said that liver biopsy findings of the malnourished infants with severe rickets showed no qualitative differences from those of the children with nutritional dystrophy without severe rickets.

In 1903 Fujinami[6] found a gross fatty liver on autopsy of a girl, about 11 years, who had been fed on high-carbohydrate-low-protein diet and developed severe rickets.

According to Nakamura,[7] fatty infiltration, congestion, infiltration of cells in the lobules or in the portal tracts, hyperplasia and hypertrophy of Kupffer's cells were found in the autopsied livers of the infants whose disease had been diagnosed as rickets with starch injury.

Okamoto[8] reported that fatty infiltration and congestion of the liver were found in experimental rickets of young rabbits.

Prof. Sano,[9] Tohoku University, made clinical and radiological examinations upon over 30,000 infants in Tohoku district, and found that malnutrition was frequently found among infants in rural villages and that a considerable proportion of these infants were suffering from florid rickets and that disturbance in liver functions was revealed in these malnourished infants with rickets.

Gillman and Gillman[10] autopsied 150 cases of African infants with malnutrition (infantile pellagra) and found that severe rickets was re-cog nized in over 50% of cases under one year of age and in 38% of cases with the age of from one to two years. In the majority of the autopsied livers, they found marked fatty infiltration.

In my present investigation, fatty infiltration, cloudy swelling, plant cell-like appearance of hepatic cells and necrosis were identified in the biopsied specimens of the livers from the malnourished infants with severe rickets, and these lesions are in my own opinion ascribable to the malnutrition especially due to high-carbohydrate-low-protein diet, as was discussed in my previous paper.

Differences in histopathological findings of the lesions of the malnourished infants from the view point of age:—All the 22 cases examined are divided into 3 groups according to their age; Group I consists of 5 cases under the age of 12 months; Group II, of 10 cases of the age from 13 to 24 months, and Group III, of 7 cases of the age from 27 to 39 months. Tables II, III and IV show the frequency of three kinds of liver changes —fatty infiltration, cloudy swelling and plant cell-like appearance of hepatic cells—in each group. A glance over these tables will give the impression that fatty infiltration occurred most frequently in Group I,

TABLE II

Incidence in Fatty Infiltration of Hepatic Cells in Groups I, II and III

Fatty infiltration \ Group	I	II	III
+	4	6	2
−	1	4	5
Total number of cases	5	10	7

TABLE III

Incidence of Cloudy Swelling of Hepatic Cells in Groups I, II and III

Cloudy swelling \ Group	I	II	III
+	3	7	4
−	2	3	3
Total number of cases	5	10	7

TABLE IV

Incidence of Plant cell-like Appearance of Hepatic Cells in Groups I, II and III

Plant cell-like appearance \ Group	I	II	III
+	1	5	6
−	4	5	1
Total number of cases	5	10	7

cloudy swelling in Group II and plant cell-like appearance of hepatic cells in Group III. And at least the incidence in plant cell-like appearance of hepatic cells in each Group (according to the age of the infants) is statistically significant. So it will be said that plant cell-like appearance of hepatic cells occur more frequently in Group III than in Groups I and II, and that fatty liver may occur oftener in Group I than in Group III, and cloudy swelling oftener in Group II than in the other groups.

Gillman and Gillman[10] found in the biopsied liver of African pellagrins that fatty liver occurred more frequently in infants than in adults,

while plant cell-like appearance of hepatic cells was, as they state, found more frequently in adults than in infants.

This tendency to develop fatty liver more easily in infants is observed in the results of my present investigation as well as in those of my previous report.[1] In a word, the younger the infants the more frequently fatty liver may occur, and the older the infants the more frequently plant cell-like appearance of hepatic cells in the biopsied livers of malnourished infants. Cloudy swelling may occur the most frequently in Group II among three groups. The differences of incidence in these three kinds of hepatic changes may be due to the differences in age of these malnourished infants.

Summary

In 22 biopsied liver specimens of malnourished infants with severe rickets, fatty infiltration was found in 12 cases, cloudy swelling in 14 cases, plant cell-like appearance of hepatic cells in 12 cases and zonal necrosis in 3 cases.

These histopathological findings showed no qualitative differences from those obtained from children with nutritional dystrophy without severe rickets.

Fatty liver was found more frequently in younger infants than in the older, and plant cell-like appearance of hepatic cells more frequently in older infants than in the younger.

Conclusions

Liver biopsy finding of malnourished infants with severe rickets is not different qualitatively from that of those infants without same, so that the liver finding identified is to be ascribed to malnutrition alone.

Fatty liver occurs probably more frequently in younger infants, while plant cell-like appearance is seen in older infants.

This work was carried out through a Grant in aid for Scientific Developmental Research from the Ministry of Education. Prof. Ts. Arakawa.

References

1) Sato, Liver Biopsy Findings of Children with Nutritional Dystrophy, in press in this Journal.

2) Bicknell & Prescott, The Vitamins in Medicine, 2nd ed., William Heinemann-Medical Books, London, 1948, 671.

3) Arakawa, Wada & Takahashi, Tohoku J. Exp. Med., 1951, **55**, 61.

4) Masuda & Aoyama, *ibid.*, 1951, **55**, 1.

5) Arakawa, Shonika Rynsho, 1953, **6**, 146.

6) Fujinami, Tokyo Igakkai Zasshi, 1903, **17**, 1059.

7) Nakamura, Chiba Igakkai Zasshi, 1937, **15**, 1215.
8) Okamoto, Nisshin Igaku, 1925, **14**, 1563.
9) Sano, Acta Pediatrica Japonica, 1954, **58**, 733.
10) Gillman & Gillman, Perspectives in Human Malnutrition, the first ed., Grune & Stratton, New York, 1951, 897.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

On "A Method of Measuring the Volume of the Circulating Blood in the Lungs and the Minute Volume by Means of Sodium Para-aminohippurate" (A Correction)

By

Takashi Yamaguchi and **Yasuhiko Hino**
(山口 隆) (日野泰賢)

From the Medical Clinic of Prof. F. Nakazawa, Tohoku University, Sendai

(Received for publication, August 16, 1956)

In the previous report[1] "A Method of Measuring the Volume of the Circulating Blood in the Lungs and the Minute Volume by Means of Sodium Para-aminohippurate", we calculated the minute volume of plasma (M) and the volume of the circulating plasma in the lungs (Q) by using the value of renal plasma flow (v). But, when the excretion rate of PAH in the kidneys is p, we can get M and Q by using the value of pv, and it is not necessary to get v.

In order to get the formula, with which pv is calculated, 0.88 v in the previous report[1] must be substituted by pv, and consequently the following formula is obtained instead of that, with which v is calculated.

$$Pv=\frac{\frac{2.303}{t_1}\log_{10}\frac{C_0}{C_1}}{\frac{C_0-C_1}{x_1}}$$

The values of pv can be got by multiplying v in the table by 0.88.

To get the formula, with which M is calculated, 0.88 v and 0.12 v must be substituted by pv and (1-p)v respectively in the previous report.[1]

And to get the one, with which Q is calculated, 0.88 v and 0.12 v must be similarly substituted by pv and (1-p)v respectively, and the same formula as described in the previous report[1] can be derived.

Thus, the values of M and Q are the same as was shown in the table, and it is clear, that our method of measuring the volume of the circulating blood in the lungs and the minute volume can be applied generally, whatever the excretion rate of PAH in the kidneys is.

References

1) Yamaguchi & Hino, Tohoku J. Exp. Med., 1955, **62**, 243.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Acute Enlargement of the Liver and Biopsy Finding: Cases of Bacillary Dysentery and Those of Nutritional Dystrophy with Acute Infections in Children. Studies on the Nutrition of Children at Hirosaki Area

(40th Report)

By

Yuji Sato
(佐藤 雄治)

From the Department of Pediatrics, School of Medicine, Hirosaki University, Hirosaki. Director: Prof. Ts. Arakawa

(Received for publication, September 22, 1955)

In 1949 Arakawa and Tachibana[1] reported two cases of secondary pellagra followed by measles, in which acute enlargement of liver was observed in the recovery stage of illness. Diarrhea, profuse sweating over the skin of upper abdomen and an increase in intensity of urinary indigo-red reaction occurred simultaneously with this acute enlargement of liver. Histopathological examinations of the liver were not undertaken at that time.

In the present paper five cases with such an acute hepatic enlargement will be reported with histological findings of the livers obtained by needle biopsy.

Method

In this paper the discription: *the liver was palpable* 3 cm. means " the liver was palpable 3 cm. below the costal margin on the right nipple line."

The procedure of liver biopsy and the discription of histological findings of biopsied specimens were the same as those given in my preceding paper.[2]

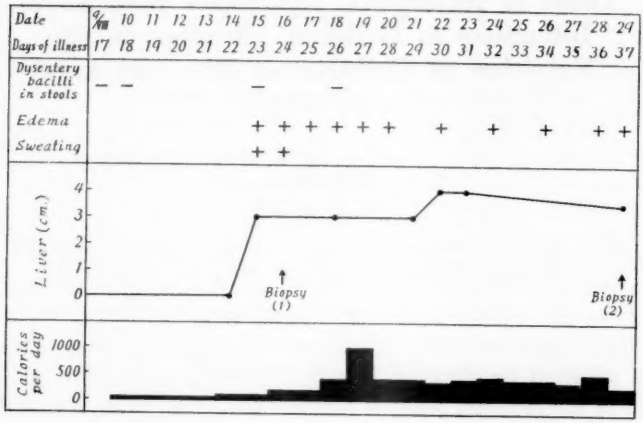
Report of Case

The cases were divided into two groups (Groups A and B): Group A included two cases of bacillary dysentery, and Group B three cases of nutritional dystrophy with acute infections.

Group A: Case of bacillary dysentery.

Case 1. Bacillary dysentery (Cf. Fig. 1). K. E., a girl of 2 years was admitted to our Hospital on 26/VII, 1951, with complaints of fever, vomiting and diarrhea for two days prior to her entrance. The patient was a malnourished girl with angular stomatitis and hypertrophy of lingual papillae, and

Fig. 1. Case 1 K.E. 2y.0m. Clinical Course.



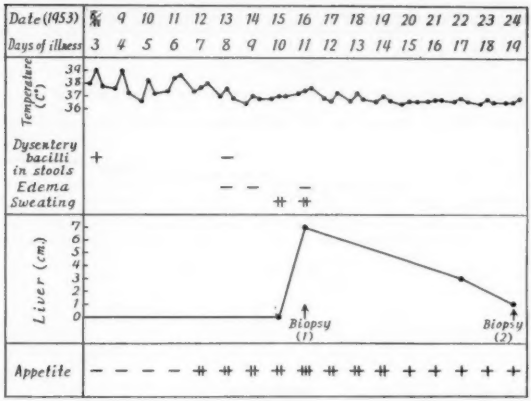
her facial expression was apathetic. The liver was not palpable. Dysentery bacilli were positive in the stool culture on 26/VII. Sulfathiazol, in the amount of 1.5 g. a day, was given orally during 26/VII～10/VIII; streptomycine, in the amount of 0.5 g. a day, orally during 16/VIII～26/VIII; and colistin (a preparation of antibiotics, Lion Seiyaku Co., Tokyo, Japan), in the amount of 500,000 units a day, orally during 26jVII～1/VIII. Ringer's solution, in the amount of 800 cc. a day, was given subcutaneously during 26/VII～7/VIII, and 20% glucose solution, in the amount of 50 cc. a day, intravenously during 26/VII～4/IX. Blood transfusion, in the amount of 50 cc. a day was given during 1/VIII～8/VIII and then during 14/VIII～26/VIII. Dysentery bacilli became negative in stool culture from 4/VIII on. The appetite increased gradually from 13/VIII on, even though caloric intake was very low, because the diet consisted of mainly rice gruel and of small amount of egg, fish and vegetable. The liver, which had remained inpalpable until 14/VIII, became palpable 3 cm. on 15/VIII.

Profuse sweating over the skin of upper abdomen, accompanied by miliaria crystallina, was observed during 15/VIII～16/VIII. From 15/VIII on the liver remained palpable 3–4 cm. and edema was found on the legs.

Liver biopsy findings were as follows: (1) fatty infiltration on 16/VIII, and (2) plant cell-like appearance of the hepatic cells with diminished fatty infiltration on 29/VIII.

Case 2. Bacillary dysentery (Cf. Fig. 2). W. K., a boy of 2 years and 10 months, was admitted to our Hospital on 8/VI, 1951, with complaints of

Fig. 2. Case 2 W. K. 2y. 10m. Clinical Course.



fever, vomiting and bloody stools for 2 days prior to his admission. He showed sign of dehydration with angular stomatitis on both sides. The liver was not palpable. Dysentery bacilli were cultured in the stools on 8/VI. Chloromycetin palmitate, in the amount of 1000 mg. a day, was given orally during 8/VI～15/VI, and sulfathiazol, in the amount of 2 g. a day, orally during 8/VI～16/VI. Ringer's solution, in the amount of 800 cc. a day, was given subcutaneously during 8/VI～20/VI.

Dysentery bacilli in stool culture became negative from 13/VI on and his appetite increased suddenly on 12/VI and became better ever since. The liver, which remained inpalpable until 15/VI, became palpable 7 cm. on 16/VI, though it gradually became less swollen and palpable only 1 cm. on 24/VI.

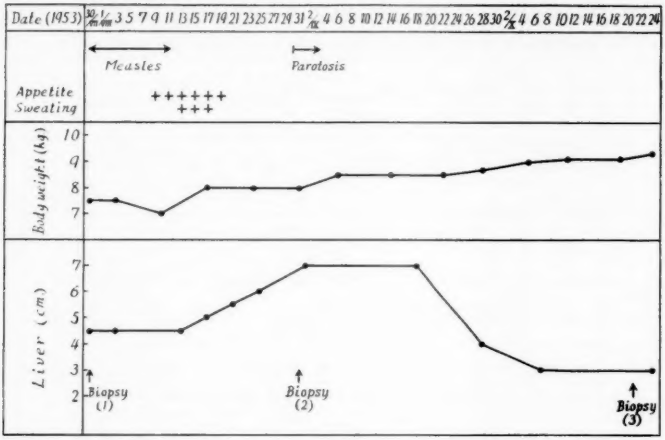
Profuse sweating over the skin of upper abdomen was noticed during 16/VI～17/VI, but edema was not observed throughout his whole stay in the hospital.

Biopsy of the liver revealed plant cell-like appearance of hepatic cells on 16/VI and cloudy swelling with small areas of plant cell-like appearance of hepatic cells on 24/VI.

Group B. Nutritional dystrophy with acute infections.

Case 3. Nutritional dystrophy with measles (Cf. Fig. 3). F. S., a girl of 2 years and 9 months was admitted to our Hospital on 15/VII, 1953, with a complaint of emaciation. Her family was in a very poor economic condition. When an infant, the patient was fed chiefly on rice gruel with small amount of goat's milk. She was a very poorly nourished girl, only 7.0 kg. in body weight. Angular stomatitis, cheilosis, hypertrophy of lingual papillae and hyperpigmentation of the skin were evident. The cupping of the end of radius was recognized on the roentgenograms of the wrists. The liver was palpable 4.5 cm.

Fig. 3. Case 3 F.S. 2y.9m. Clinical Course.

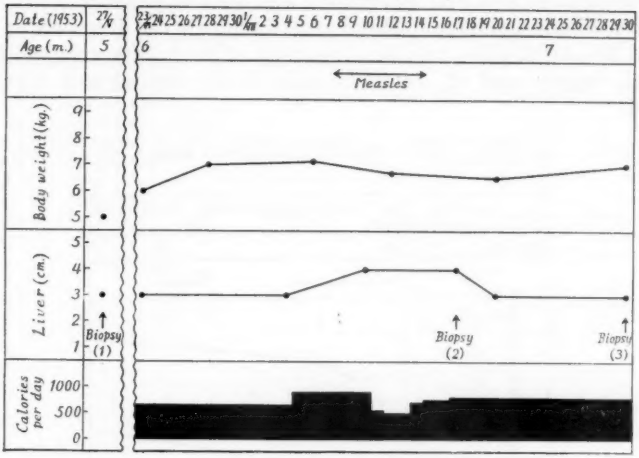


Fever and coughing were complained of on 30/VII and thereafter, and measles exanthema developed on 3/VIII. Beginning with 9/VIII her temperature began to return to normal and her appetitie was markedly increased, body weight beginning to increase. The liver was gradually increased in its size and became 6 cm. on 25/VII, and 7 cm. on 1/VIII, but it began to decrease on 19/VIII and thereafter; it was palpable only 3 cm. during 7/X~29/X. Profuse sweating over the skin of upper abdomen, was accompained by miliaria crystallina, was observed during 13/VIII~17/VIII. An enlargement[3] of parotid glands was observed on both sides from 30/VIII on. On 27/VIII, riboflavin content of blood[4][5][6] was 4.8 γ%, protein bound iodine[7] of serum was 5.15 γ% and E K G[8] showed a prolongation of the Q-T intervals and the flat T waves. All these findings were frequently observed in children with nutritional dystrophy at Hirosaki area.

Liver biopsies were carried out three times and the histological findings were as follows; (1) on 30/VII, plant cell-like appearance of hepatic cells was found in a marked degree; (2) on 31/VIII, such an appearance of hepatic cells was found in a less degree, but fibrosis was identified as an additional finding; and (3) on 23/X, swelling of hepatic cells was slightly diminished and stainable substances in the protoplasma of hepatic cells were increased—in a word, the finding of cloudy swelling in a mild defree—and fibrosis remained in the same degree as found on 31/VIII.

Case 4. Nutritional dystrophy with measles (Cf. Fig. 4). M. T., a boy of 5 months was admitted to our Hospital on 27/V, 1953, for the purpose of isolation from his mother who had been suffering from pulmonary tuberculosis. The patient was born in maturity and was mainly fed on rice gruel with small amount of cow's milk, due to a poor secretion of mother's milk. He was poorly

Fig. 4. Case 4 M.T. 5m. Clinical Course.



nourished and his lingual papillae were hypertrophied. Subcutaneous tissues were flabby on palpation and the liver was palpable 3 cm. Tuberculin test with 1 :2000 old tuberculin remained negative during his stay in the Hospital. His appetite was fairly good and the average intake of food amounted to about 120 Calories per day per kg. of body weight. From 5/VII on his body weight began to increase and the liver was increased in its size. An attack of measles during 8/VII～14/VII resulted in a temporary decrease in appetite and in body weight. From 20/VII on, the liver became less swollen until it stayed to the size: palpable 3 cm.

Liver biopsies were carried out three times and the histological findings were as follows: (1) On 27/V, fatty infiltration was found in a marked degree: (2) on 17/VI, instead of fatty infiliration, plant cell-like appearance of the hepatic cells was identified as a prominent feature and fibrosis was evident; and (3) on 30/VI, cloudy swelling was observed in a mild degree.

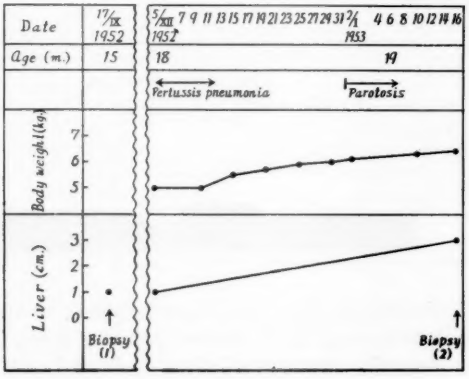
Case 5. Nutritional dystrophy with pertussis pneumonia (Cf. Fig. 5). Y. K., a boy of 15 months visited our out-patient Department on 17/IX, 1952, with complaints of fever and coughing. At that time his affection was diagosed as " bronchopneumonia and nutritional dystrophy[9][10] with severe rickets," and liver biopsy was performed.

On 5/XII, 1952, he was admitted to our Hospital under the diagnosis of purtussis pneumonia. The patient was a malnourished infant and showed angular stomatitis, cheilosis, swelling of lingual papillae, and deformities of thorax and of spine. His body weight was only 5 kg. Roentgenogram of the wrist showed cupping of radius. Pertussis pneumonia was gradually suppressed by the treatment with chloromycetine and with penicillin, but an increase in body weight was far from being stisfactory; he was only 6.4 kg. at the age of

19 months (on 14/I, 1953).

On 16/I, 1953, the liver was swollen and became palpable 3 cm., and the second liver biopsy was made. Histological findings of the liver were as follows: (1) on 17/IX, 1952, plant cell-like appearance of the hepatic cells was found in a marked degree; and (2) on 16/I, 1953, cloudy swelling and fibrosis were identified.

Fig. 5. Case 5 Y. K. 15 m. Clinical Course.



Comment

The liver biopsy findings were summarized in Table I (Cf. Table I).

In Group A (cases of bacillary dysentery), the liver was not palpable on the day of admission, but it was markedly increased in its size and became palpable 3–7 cm. At the stage of the acute hepatic enlargement, liver biopsy revealed a fatty infiltration in Case I and a plant cell-like appearance of hepatic cells in Case 2.

In Group B (cases of nutritional dystrophy with an actue infection) the liver was already palpable on the day of admission and the first liver biopsy was performed. During hospitalization, an increase in liver size was observed and the second biopsy was made at this stage. Liver biopsy findings were as follows: (1) Cloudy swelling and fibrosis were found on the second biopsy of Cases 3 and 5 instead of the plant cell-like appearance of hepatic cells as seen on the first biopsy; and (2) plant cell-like appearance of hepatic cells on the second biopsy of Case 4 instead of the fatty infiltration as seen on the first biopsy.

Profuse sweating over the skin of upper abdomen, accompanied by miliaria crystallina, was found in 3 cases (Cases 1, 2 and 3) out of the 5 cases. It was observed almost simultaneously with the occurrence of acute enlargement of liver.

TABLE I

Liver Biopsy Findings of the Cases in which Acute Enlargement of the Liver was Observed

Group	No. of case	Whether or not the liver was palpable before acute enlargement of the liver developed ?	Histological findings of the biopsied liver		
			Before onset of acute enlargement	Stage of acute enlargement	Later stage
Group (A) Bacillary dysentery	1	−	(0 cm.)*	(3 cm.)* Fatty infiltration	(3.5 cm.)* Plant cee-like appearance
	2	−	(0 cm.)*	(7 cm.)* Plant cell-like appearance	(1 cm.)* Cloudy swelling
Group (B) Nutritional dystrophy with acute infections	3	+	(4.5 cm.)* Plant cell-like appearance	(7 cm.)* Cloudy swelling+ fibrosis	(3 cm.)* Cloudy swelling+ fibrosis
	4	+	(3 cm.)* Fatty infiltration	(4 cm.)* Plant cell-like appearance	(3 cm.)* Cloudy swelling+ fibrosis
	5	+	(1 cm.)* Plant cell-like appearance	(3 cm.)* Cloudy swelling+ fibrosis	

* 0 cm., 3 cm., 7 cm., etc. . . . show that the liver was palpable. 0 cm., 3 cm., 7 cm., etc. below the costal margin.

In the later course of Cases 2, 3 and 4, a decrease in liver size was found and a swelling of hepatic cells was diminished in the biopsied specimens examined at this stage.

In 1920, Saito[11] reported that hepatic enlargement was occasionally observed in the course of infantile diarrhea or of dysentery in children, and he supposed that this hepatic enlargement might result from congestion, or a ptosis of the liver, or more probably from a swelling of the hepatic cells, but he did not perform any liver biopsy.

In 1929, Shizume *et al.*[12] reported that hepatic enlargement in the course of infantile diarrhea was observed in 37% of 78 cases examined, and that fatty infiltration of liver was revealed in all the 5 cases with hepatic enlargement which were autopsied.

In 1936, Ohta[13] reported that hepatic enlargement was occasionally observed in the recovery stage and in the critical condition of infantile diarrhea.

In 1952, Gomez *et al.*[14] reported, basing upon their liver biopsy findings, that hepatomegaly was observed as one sign of ' nutritional recovery syndrome ' from infantile pellagra among Mexican children.

and that the hepatomegaly more frequently resulted from protein-depletion-lesion[15] of the liver (causing a plant cell-like appearance of hepatic cells)[16] than from fatty infiltration of the organ, and that along with the hepatomegaly there was distension of the abdomen, a thoraco-abdominal venous network and ascites.

Profuse sweating over the skin of upper abdomen, which was frequently observed simultaneously with the occurrence of hepatic enlargement, was, as has been mentioned, found in 3 cases out of 5 cases of my own investigation, but it was not described in the report of Gomez *et al.*[14]

According to my present investigation of 5 cases with acute hepatic enlargement livers, fatty infiltration was found in 1 case (Case 1), plant cell-like appearance of hepatic cells in 2 cases (Cases 2 and 4) and cloudy swelling with fibrosis in the other remaning 2 (Cases 3 and 5).

Summary

In the course of bacillary dysentery of two children, acute enlargement of liver was observed. Liver biopsy revealed fatty infiltration in one case and plant cell-like appearance of hepatic cells in the other.

In three cases of nutritional dystrophy with acute infections, an increase in the size of liver was observed and this increase was, as was revealed by liver biopsy, due to cloudy swelling with fibrosis in 2 cases and to plant cell-like appearance of hepatic cells in the other.

Almost simultaneously with the occurrence of the hepatic enlargement in question, profuse sweating over the skin of upper abdomen was found in three out of the five cases with acute enlargement of liver.

Conclusions

On the basis of liver biopsy of actue enlargement of the liver in children, the following conclusions will be made:

1. Acute enlargement of the liver in recovery stage of bacillary dysentery, during which the patients would like to take still a low-protein-high-carbohydrate diet, on which they had been fed during the acute stage, for a considerably long period, was due to fatty infiltration or plant cell-like appearance of hepatic cells, and these changes in the liver were the same as seen in the case of severe nutritional dystrophy.

2. Acute enlargement of the liver in recovery stage of acute infection which occurred in cases with nutritional dystrophy resulted chiefly from cloudy swelling and fibrosis, and these changes in the liver were observed in cases with a nutritional dystrophy which was mild but of relatively long duration.

This work was carried out through a Grant in aid for Scientific Develop-

mental Research from the Ministry of Education. Prof. Ts. Arakawa.

References

1) Arakawa & Tachibana, Hirosaki Igaku, 1950, **1**, 56.
2) Sato, Liver Biopsy Findings of Children with Nutritional Dystrophy, in press in this Journal.
3) Sato, Enlargement of Partotid glands and Its Histological Findings, to be published in a coming issue of this Journal.
4) Kagaya, Vitamins (Japanese), 1953, **6**, 777.
5) Kagaya, *ibid.*, 1954, **7**, 557.
6) Kagaya, *ibid.*, 1954, **7**, 563.
7) Hashimoto, Protein Bound Iodine Level of Sera of Children with Nutritional Dystrophy, in press in this Journal.
8) Ishioka, Electrocardiograms of Children with Nutritional Dystrophy, read at 56th General Meeting of the Japanese Pediatric Society, April, 1954.
9) Arakawa, Wada & Takahashi, Tohoku J. Exp. Med., 1951, **55**, 61.
10) Masuda & Aoyama, *ibid.*, 1951, **55**, 1.
11) Saito, Zika Zasshi, 1920, No. 238, 301.
12) Shizume, Okubo & Tada, *ibid.*, 1929, No. 345, 257.
13) Ohta, *ibid.*, 1936, No. 435, 1075.
14) Gomez, Galvan & Munoz, Pediatrics, 1952, **10**, 513.
15) Moore, Textbook of Pathology, Philadelphia, W. B. Saunders Company, 1944.
16) Gillman & Gillman, Perspectives in Human Malnutrition, 1st ed., Grune & Straton New York, 1951.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

On the Treatment of Muscle Atrophy with Very High Frequency Impulses

By

Yoshihiko Umehara

(梅原 芳彦)

From the Department of Internal Medicine, Sendai National Hospital, Sendai. Director: Dr. T. Kato, Professor Emeritus of Tohoku University

(Received for publication, October 13, 1955)

It is of a high clinical importance to find the best means of delaying or lessening the onset of muscle atrophy following central or peripheral motor paralysis. Various physical and chemical methods aimed for this purpose have been tried to date, with more or less success. Langley and Kato[1] performed the experiment of applying electric stimulation by means of condenser shocks to the gastrocnemius muscle group in one of the legs of rabbits with their tibial nerves on both sides severed, and found that the muscles on the side thus shock-treated were apparently better defended from atrophy than those on the other side. The rhythmic extensions of the paralyzed muscles have also been accepted as efficacious, though in a slight degree, while massage alone of the muscle was not nearly effective for the purpose. Since d'Arsonval first proved the harmless pyrogenic effect of very high frequency, the so-called d'Arsonval's short waves came into application in clinical practice. Later, Schliephake[2] succeeded him by confirming the effective biological action of very high frequency (V.H.F.); G. Takahashi, Hashimoto and T. Takahashi[3] found extension of muscles, which were exposed to such waves. In consideration of those findings, the author has undertaken the present experiment to observe the therapeutic effect of the very high frequency impulses upon the atrophic process of paralyzed muscles, using a new apparatus which was recently devised by Dr. M. Kobayashi and Dr. T. Kato.

Experimental

Method

10 rabbits of about 2 kg. each were employed. The sciatic nerves were severed on both sides, and on from the 3rd to 6th day after operation, the tibial muscles on one side began to be stimulated by placing

a pair of pole plates (6×6 cm^2) thereon of very high frequency impulse apparatus (5–IR–1001) devised by Dr. M. Kobayashi and Dr. T. Kato (1947) and constructed by Nippon Electric Co. Laboratory with the following standard: Input of 100 V A.C. and 600 W, peak pulse power of ca. 1 KW, wave length of 5 m., pulse repetition frequency of 1 cycle /sec. and ratio pulse duration/pulse repetition period of ca. $\frac{2}{100}$. The stimulation was applied for about 10 min. every day, down to from 3rd to 62nd day thereafter. Then, the gastrocnemius muscle groups on both sides were extracted from their proximal tendons down to the level of their insertion of the Achilles tendon and weighed in order to compare the progress of muscle atrophy on both sides, and also a part of the specimens were cut into tissue preparations, to examine their histological changes in comparison.

Results and Discussion

The results are shown in Table I. The gastrocnemius muscle groups

Table I

Comparison of the Muscle Weights of Treated and Non-treated Sides

Figures of the treated side in gothic type

No. of Exp.	Sex	Body weight (kg.)	Ditto at death (kg.)	Percentage loss of weight	Days after nerve section until impulse applied	Duration of treatment (days)	Weight of gastrocnemius group (g.) right	Weight of gastrocnemius group (g.) left	Percentage gain on stimulated side
1	♀	2.0	1.6	−20.0	2	3	5.65	**6.26**	+10.8
2	♂	1.9	1.7	−10.5	5	12	3.52	**4.00**	+13.6
3	♂	2.8	2.8	± 0	5	11	6.26	**7.28**	+16.3
4	♀	2.8	2.3	−17.9	4	58	2.23	**2.82**	+17.5
5	♂	2.0	1.8	−10.0	5	30	**3.60**	3.15	+14.3
6	♂	2.2	2.0	− 9.1	14	22	**3.68**	3.46	+ 6.4
7	♀	2.2	2.1	− 4.5	5	32	**4.15**	3.52	+17.9
8	♀	2.2	2.0	− 9.1	5	30	3.82	**4.27**	+11.8
9	♂	2.2	1.8	−18.2	5	62	2.00	**2.38**	+19.0
10	♂	2.0	1.8	−10.0	5	21	**3.66**	3·55	+ 9.3
Average		2.2	2.0	−10.9	6	28	3.72	**4.21**	+13.2
Standard deviation				± 4.7			±1.33	±**1.37**	± 4.1
Maximum		2.8	2.8	± 0	14	62	6.26	**7.28**	+19.0
Minimum		1.9	1.6	−20.0	2	3	2.00	**2.38**	+ 6.4

of the rabbits were treated with the impulse stimulation apparatus on one side for 3 to 62 days, for 28 days on an average, after the section of the sciatic nerves on both sides. All the rabbits, excepting only one case (No. 3) lost body weight, the mean percentage of loss in weight amounting to 10.9±4.7. The weight of the muscle group with severed nerve and not stimulated by the impulse was 6.26 g. at the maximum, 2.00 g. at the minimum and 3.72±1.33 g. in the mean, while that of the muscle group on the stimulated side amounted to 7.28 g. at the maximum, 2.38 g. at the minimum and 4.21±1.37 g. on an average, showing an excess over that on the unstimulated side of 19% at the maximum, of 6.4% at the minimum and of 13.2±4.1% on an average.

According to Langley and Kato,[1] the gastrocnemius muscle groups, when cut off from their nervous control, will begin to decrease in weight on the 3rd day after the neurectomy, losing weight rapidly thereafter, to become reduced by 30% at the end of the 2nd week. Thence, the speed of decrease slackens somewhat, but yet, at the end of the 5th week, the loss in weight is said to reach 50% of the orginal weight. In No. 1 of the present experiment, the stimulation was applied for 3 days starting on the 3rd day after the nerve cutting, and the weight of the muscle groups on the 4th day thereafter was 6.26 g. on the stimulated side and in excess of 10.8% over that of the muscle group on the untreated side, 5.65 g., suggesting that the impulse treatment was effective in suppressing the atrophy of the muscles in some extent. In No. 2, the application of impulses was begun on the 6th day and continued for 12 days. On the 13th day, the stimulated muscle group weighed 4.00 g., showing an increment of 13.6%, in comparison with the group on the other side which had the weight of 3.52 g. In No. 6, the stimulation was begun on the 15th days, when the muscle atrophy had progressed to a considerable extent, and continued for 22 days. The muscles weighed 3.68 g., while the weight of the muscles on the other side left untreated for 36 days after the nerve cutting was 3.46 g., the difference amounting only to 6.4%. Thus, when the muscle atrophy has considerably progressed by the lapse of 2 weeks, as in this case, the impulse stimulation is little effective in preventing further atrophy. In the remaining 7 cases, the stimulation was begun on the 6th day after the nerve cutting operation, that is, in the early stage of muscle atrophy, and continued for 11 to 62 days. The comparison with the control side muscles thus left untreated for 16 to 67 days after the denervation showed excess in the weight of the stimulated muscles with the difference of 9.3 to 19%, but in all cases the muscle atrophy was observed on both sides progressing in parallel, while the fibrillations, which occur continuously in the atrophying muscle as rapid rhythmic contraction of the muscle fibres, was mild on the treated side.

Histological examination of the atrophic muscle showed that in the specimens stimulated with impulse the dilatation of the capillaries was much more marked and degenerative changes of muscle fibres and fibre celles were somewhat less advanced than in the untreated groups.

Thus, it may be concluded that V.H.F. administered in impulse act in suppressing atrophy of muscles, the impulses giving rise to contraction of the muscles, which, under the action of the micro-waves, more or less extend out, the capillaries dilate and the blood circulation is expedited, thus contributing to protection of the muscles from atrophy. The treatment, however, must be begun within 2 weeks after the blocking of the nervous control and should be continued in daily administration. As for the effective period of continued treatment, the author's experiments seem to indicate that the atrophy preventing effect is notable for the first two months, but the muscle atrophy does sooner or later occur even in the treated muscles, as in untreated muscles, and it is probably impossible to prevent onset of fibrillation of muscles to some extent.

Summary

The sciatic nerves of 10 rabbits were severed on both sides and very high frequency impulses were administered to their gastrocnemius muscle groups unilaterally. In 3 to 62 days after the beginning of the stimuation, the muscles groups were cut out at the insertion of the tendons on both sides and weighed, for estimating the degree of muscle atrophy in comparison. The results obtained were as follows:

1. The weight of the gastrocnemius muscles on the treated side in comparison with that of the muscles on the other side was always larger and apparently showed that such a treatment was effective in controling muscle atrophy.

2. The impulse treatment should be begun in a short period after the cutting off of the nervous control and should be continued in daily administration more than two months.

References

1) Langley & Kato, J. Physiol., 1915, **49**, 432.
2) Schliephake, Dtsch. med. Wschr., 1932, 1235.
3) G. Takahashi, Hashimoto & T. Takahashi, Tohoku Igaku Zasshi, (Jap.), 1942. **31**, 65,

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Effect of Blood Pressure Depressants on Plasma Proteins in Hypertensive Diseases

By

Yoshihiko Umehara
(梅原 芳彦)

From the Department of Internal Medicine, Sendai National Hospital, Sendai. Director: Dr. T. Kato, Professor Emeritus of Tohoku University

(Received for publication, October 13, 1955)

Hypertension, accompanied by increase of the resistance against blood flow in arterioles due to their functional or organic change, causes serious symptoms through the reduction of blood flow in various organs, in particular, kidneys, heart, brain, and liver. On the other hand, the liver and kidneys as well as mesencephalon are known to play important parts in the metabolism of protein and the anomalies in the said metabolism in the case of functional disturbance of these organs have been brought to light by a series of researchers. Thus, it is not difficalt to surmise that, in hypertension, the protein metabolism is disturbed and certain changes are found in the protein picture of plasma. Now it is a question of great clinical interest, how the plasma protein content in hypertension is affected by artificial blood pressure fall induced by use of depressants. The author has undertaken the present experimental investigation in order to solve this problem, thus to make a contribution to the knowledge of the effect of changes in blood flow of various organs, induced by artificial blood pressure lowering, upon the protein metabolism of the organism.

Experimental

Material and Method

Taking as control 6 normotensive subjects of the age between 42 and 60 years, on an average of 52 years, the following experiments were made with 14 cases of benign essential hypertension, 8 cases of malignant essential hypertension, 5 cases of hypertensive chronic glomerulonephritis and 2 cases of acute glomerulonephritis with high blood pressure.

After stabilization of the blood pressure in rest for ca. 30 minutes, blood samples were drawn from cubital veins and hexamethonium bromide (Methobromine), veratrum viride (Trapon), or 1-hydrazinophthalazine (Apresoline) was injected intramuscularly; then, the blood

pressure was measured every 5 minutes and when it fell to the lowest, blood samples were drawn again. In the heparinized plasmas before and after the lowering of blood pressure were estimated the total protein content with Pulfrich's immerse refractometer and the protein fractions with Tiselius' apparatus of Hitachi's Type HA, using phosphoric acid buffer solution of pH 8.0 and ionic strength of 0.1, and following the standard method laid down by the Japanese Research Society of Electrophoresis. The changes of these contents were studied in collation with the control values.

Results and Discussion

In Table I the plasma protein pictures of 6 normotensive subjects and in Tab. II the same of 14 benign essential hypertension cases, before

Table I

Average Plasma Protein Picture of 6 Normotensive Subjects

	Age	Blood pressure (mm.Hg)	Total protein (g/dl)	Albumin (g/dl)	α-Globulin (g/dl)	β-Globulin (g/dl)	Fibrinogen (g/dl)	γ-Globulin (g/dl)	$\frac{A}{G}$
Average	52	138–75	8.06	4.79	0.65	0.73	0.57	1.31	1.78
Standard deviation			±0.12	±0.33	±0.13	±0.07	±0.12	±0.16	±0.18
Maximum	60	146–80	8.49	5.18	0.83	0.83	0.76	1.45	2.14
Minimum	42	126–78	7.50	4.37	0.53	0.65	0.46	1.05	1.66

and after the administration of the blood pressure depressants are listed. As are seen in the tables, in the patients of benign essential hypertension with the systolic pressure of 200 mm. Hg and the diastolic pressure of 103 mm. Hg on an average, the mean total protein (7.94 g/d*l*) is decreased approximately to the lowest limit of normal value, the mean albumin content (3.96 g/d*l*) shows a decrease, while the mean α-, β- and γ-globulins (0.71 g/d*l*, 0.76 g/d*l*. 1.36 g/d*l*, resp.) are increased nearly to the upper limit of the normal, fibrinogen (1.11 g/d*l*) shows a marked increase, and accordingly $\frac{A}{G}$ ratio (1.39) is lowered. At the time of lowest pressure following the use of the depressants, with the systolic pressure of 161 mm. Hg and the diastolic pressure of 92 mm. Hg in mean, the total protein rose by 0.4% on an average, also albumin was increased by 4.0%, each approaching the normal value, while α- and β-globulin were decreased by 16.9 % and 7.9% respectively, falling below the normal values, and fibrinogen, γ-globulin and $\frac{A}{G}$ were increased by 0.9%, 5.9% and 6.5%. Thus, fibrin-

ogen further rose above, γ-globulin came up near to the upper limit of, and ratio $\frac{A}{G}$ approached the normal values.

Data in 8 cases of malignant hypertension are shown in Tab. III. In mean, the total protein (7.62 g/d*l*), albumin (3.60 g/d*l*) and ratio $\frac{A}{G}$ (1.23) are markedly decreased below the normal values, while α-(0.77 g/d*l*), β-(0.78 g/d*l*) and γ-globulins (1.37 g/d*l*, all in mean) show values near the upper limit of the normal, fibrinogen (1.09 g/d*l*) having a value much higher than the normal. After the administration of the drugs, the mean systolic blood pressure falling from 218 mm. Hg to 161 mm. Hg and the diastolic from 109 to 91 mm. Hg, the total protein, albumin, α- and β-globulins and the $\frac{A}{G}$ ratio showed decrease of 0.9%, 10.3%, 9.1% and 19.2%, respectively, thus the globulins only remaining in the range of normal values, while the other values falling below normal; fibrinogen was markedly increased over the normal values and γ-globulin showed also a rise.

In 5 cases of chronic glomerulonephritis with the systolic blood pressure of 185 mm. Hg and the diastolic of 105 mm. Hg on an average (Tab. IV , the total protein, albumin, β-globulin, fibrinogen and $\frac{A}{G}$, having the values of 7.38 g/d*l*, 3.43 g/d*l*, 0.97 g/d*l*, 1.03 g/d*l* and 1.17 in mean respectively, are subnormal,· while α- and γ-globulins are nearly normal, each with the mean values of 0.63 g/d*l* and 1.31 g/d*l*. After the administration of the depressants systolic blood pressure falling to 162 mm. Hg and the diastolic to 94 mm. Hg on an average, the total protein, albumin, α- and γ-glublins and $\frac{A}{G}$ were decreased by 2.6%, 7.0%, 11.1%, 4.6% and 1.7% respectively, thus only the values of α- and γ-globulins remaining in the range of the normal, but the other values falling even more subnormal, while β-globulin and fibrinogen increased further above the normal by 1.0% and 11.7% respectively.

In 2 cases of acute glomerulonephritis (Tab. IV), in which prior to the lowering of the blood pressure, the mean systolic pressure stood at 182 mm. Hg and the diastolic at 90 mm. Hg, the total protein, albumin, α- and β-globulins, fibrinogen, γ-globulin and $\frac{A}{G}$ showed the values of 6.74 g/d*l* (perceptibly subnormal), 2.95 g/d*l* (ditto), 0.93 g/d*l* (supernormal), 0.72 g/d*l* (normal), 0.74 g/d*l* (supernormal), 1.37 g/d*l* (normal), and 0.98 (perceptbly subnormal), respectively. After the administration of the depressants, the systolic blood pressure fell to 155 mm. Hg and the di-

TABLE

Plasma Protein Fractions of Benign Essential Hypertension

Case	Sex	Age	K.W. Eye change	Protein-uria	Depressants (mg)	Blood pressure before & after depressants (mm/Hg)	Total protein g/dl	Total protein Change %
1	♂	52	II	±	C_6 (25)	b. 198– 90 a. 108– 70	8.37 7.96	−4.9
2	♂	53	II–III	–	C_6 (25)	b. 218–118 a. 200–100	7.42 7.42	± 0
3	♂	53	II–III	–	C_6 (25)	b. 164– 85 a. 138– 88	7.74 7.76	+0.3
4	♂	56	II	–	C_6 (25)	b. 200– 78 a. 164– 95	7.69 7.98	+3.8
5	♂	57	II	–	Trapon (0.75)	b. 200–112 a. 164– 90	7.71 7.71	± 0
6	♂	62	II	–	Trapon (0.75)	b. 200– 62 a. 175– 58	7.63 7.70	+9.2
7	♀	52	II–III	–	Trapon (0.75)	b. 195–105 a. 178–100	8.49 8.71	+2.6
8	♂	47	II	–	Trapon (0.8)	b. 220–122 a. 208–125	7.20 7.33	+1.8
9	♂	54	II	–	Trapon (0.75)	b. 210–110 a. 170– 95	8.71 8.71	± 0
10	♀	52	II	–	Apresoline (20)	b. 218–115 a. 180–100	8.49 8.71	+2.6
11	♀	50	II–III	±	Apresoline (20)	b. 210–115 a. 135– 90	8.28 8.06	−2.7
12	♀	54	II–III	±	Apresoline (20)	b. 198–110 a. 134– 80	8.49 8.61	+1.4
13	♀	67	II	–	Apresoline (20)	b. 190–115 a. 158–104	7.49 7.49	± 0
14	♀	48	II	–	Apresoline (15)	b. 182–104 a. 144– 98	7.38 7.45	+0.9
Average						b. 200–103 a. 161– 92	7.94 7.97	+0.4

astolic to 62 mm. Hg in the mean; total protein was decreased by 3.1%, further descending subnormal, albumin reduced by 5.1%, also further subnormal, while α- and β-globulins remained nearly unchanged, fibrinogen increased by 5.4%, slightly above normal and γ-globulin decreased by 5.1%, but within the normal range and $\frac{A}{G}$ decreased by 3.1% further subnormal.

In summary, the total protein content in plasma usually lies below the normal level in hypertension, the decrement being largest in acute glomerulonephritis followed by chronic glomerulonephritis, malignant

II

Before and After the Administration of Depressants

Albumin		α-globulin		β-globulin		Fibrinogen		γ-globulin		$\frac{A}{G}$	
g/dl	Change %	g/dl	Change %	g/dl	Change %	g/dl	Change %	g/dl	Change %		Change %
4.76	− 2.3	0.67	−34.3	0.78	− 2.6	0.84	− 9.5	1.32	+ 0.8	1.72	−29.7
4.65		0.44		0.76		0.76		1.33		1.21	
3.74	+ 0.5	0.82	− 8.5	0.72	− 9.7	1.28	− 3.1	0.87	+ 8.0	1.55	+ 1.3
3.76		0.75		0.65		1.24		0.94		1.57	
3.87	+ 3.4	0.79	−20.3	0.89	−10.1	1.09	± 0	1.09	+13.8	1.40	+ 7.1
4.00		0.63		0.80		1.09		1.24		1.50	
4.08	+ 5.1	0.52	−28.8	0.60	−46.7	0.86	+39.5	1.47	+ 0.7	1.55	+27.7
4.29		0.37		0.32		1.52		1.48		1.98	
3.93	+ 0.8	0.61	− 6.6	0.61	−27.9	0·92	+14.1	1.63	+ 3.7	1.37	+ 7.3
3.96		0.57		0.44		1.05		1.69		1.47	
3.63	+19.8	0.58	− 5.2	0.58	−13.8	1.49	−46.3	1.34	+11.9	1.45	+17.9
4.35		0.55		0.50		0.80		1.50		1.71	
4.09	+ 3.2	0.83	− 9.6	0.77	+14.3	1.21	+25.6	1.60	+ 6.3	1.28	− 0.8
4.22		0.75		0.88		1.52		1.70		1.27	
3.23	+20.4	0.89	−64.0	0.47	−12.8	1.04	+ 8.7	1.57	+ 0.6	1.11	+51.3
3.89		0.32		0.41		1.13		1.58		1.68	
4.64	+ 5.6	0.83	− 7.2	0.92	− 6.5	0.65	+ 6.2	1.66	−10.8	1.36	+16.2
4.90		0.77		0.86		0.69		1.48		1.58	
4.32	+ 9.5	0.90	−15.6	0.86	−12.8	1.02	−21.1	1.38	+ 8.0	1.38	+ 8.0
4.73		0.76		0.97		0.80		1.49		1.49	
4.00	− 8.3	0.58	− 1.7	0.72	− 5.6	1.63	+ 3.7	1.32	+ 3.8	1.47	− 7.5
3.67		0.57		0.76		1.69		1.37		1.36	
3.84	− 1.3	0.46	−17.4	1.12	− 3.6	1.48	+ 2.7	1.10	+60.0	1.21	− 2.5
3.79		0.38		1.08		1.52		1.76		1.18	
3.80	+ 1.8	0.67	− 6.0	0.56	− 5.4	1.06	+ 4.7	1.40	− 2.9	1.44	+ 6.9
3.87		0.63		0.53		1.11		1.36		1.54	
3.48	+ 2.6	0.79	− 2.5	0.90	− 7.8	0.90	+ 3.3	1.31	− 3.1	1.16	+ 6.9
3.57		0.77		0.83		0.93		1.27		1.24	
3.96	+ 4.0	0.71	−16.9	0.76	− 7.9	1.11	+ 0.9	1.36	+ 5.9	1.39	+ 6.5
4.12		0.59		0.70		1.14		1.44		1.48	

and benign essential hypertension in the order named. When the blood pressure is lowered following administration of depressants, the total protein content rises to approach the normal in benign essential hypertension, but in the other forms, it falls further below normal, the fall being most marked in acute, next in chronic nephritis and lightest in malignant hypertension. Albumin content also shows decrease in general, in the order of acute and chronic nephritis and malignant and benign essential hypertension. Upon effected lowering of the blood pressure by administration of the drugs, it increases to approach the normal level in benign essential hypertension, but falls further below the normal in the other

TABLE

Plasma Protein Fractions of Malignant Essential Hypertension

Case	Sex	Age	K.W. Eye change	Protein-uria	Depressants (mg.)	Blood pressure before & after depressants (mm.Hg)	Total protein g/dl	Total protein Change %
1	♀	63	III	±	C_6 (37.5)	b. 210–115 a. 135– 90	7.44 7.32	−1.6
2	♀	67	III–IV	++	C_6 (25)	b. 160– 82 a. 132– 85	7.20 7.42	+3.1
3	♂	54	III–IV	+	Trapon (0.6)	b. 280–135 a. 200–110	9.07 8.38	−7.6
4	♂	54	III–IV	+	Trapon (2.0)	b. 237–120 a. 135– 85	7.69 7.65	−0.5
5	♂	74	III–IV	##	Trapon (0.68)	b. 190– 90 a. 160– 80	7.63 7.76	+1.7
6	♀	63	III	±	Apresoline (20)	b. 198–110 a. 136– 80	6.70 7.04	+5.1
7	♂	65	III–IV	##	Apresoline (20)	b. 190– 80 a. 138– 70	7.36 7.21	−2.0
8	♂	67	III–IV	++	Apresoline (12)	b. 280–140 a. 254–125	7.85 7.65	−2.5
Average						b. 218–109 a. 161– 91	7.62 7.55	−0.9

forms, most strikingly in malignant essential hypertension, followed by chronic and acute nephritis. α-globulin is a little supernormal in acute nephritis, but in the other cases, it is within the range of normal values; when the blood pressure is artificially reduced, it tends to fall except in acute nephritis, in the order of benign essential hypertension, chronic nephritis and malignant essential hypertension. Also β-globulin is somewhat supernormal in chronic nephritis, while in the other forms it is within the range of normal values. After the administration of depressants, it shows no change in either direction in nephritic hypertension; in essential hypertension there is apparently a tendence to decrease, but not below normal range, the reduction being more perceptible in malignant than in benign form. Fibrinogen is found increased in all cases over the normal level and rises even further upon treatment with depressants, the increment being largest in malignant essential hypertension, next in chronic nephritis, then in acute nephritis and smallest in benign hypertension. γ-globulin is in normal range in all forms, but upon artificial lowering of blood pressure, tends to decrease in nephritic and to increase in essential hypertensions, showing a particular rise over the range of normal values in malignant essential hypertension. Albumin-globulin ratio $(\frac{A}{G})$ shows

III

Before and After the Administration of Depressants

Albumin		α-globulin		β-globulin		Fibrinogen		γ-globulin		$\frac{A}{G}$	
g/dl	Change %	g/dl	Change %	g/dl	Change %	g/dl	Change %	g/dl	Change %		Change %
3.50	−10.9	0.77	−14.3	0.77	−20.8	1.08	+20.4	1.34	+19.6	1.22	−10.7
3.12		0.66		0.61		1.30		1.60		1.09	
3.28	+ 0.9	0.74	± 0	0.81	− 2.5	1.11	+ 8.1	1.26	+10.3	1.17	− 3.4
3.31		0.74		0.79		1.20		1.39		1.13	
4.81	− 1.9	0.88	− 4.5	0.74	− 2.7	1.08	+12.0	1.56	+ 8.3	1.51	−20.5
3.92		0.84		0.72		1.21		1.69		1.20	
3.62	−10.0	0.80	−13.8	0.79	−20.3	1.12	+20.5	1.38	+21.0	1.22	−10.7
3.26		0.69		0.63		1.35		1.67		1.09	
3.72	− 8.6	0.60	−20.0	0.86	−22.1	1.00	+15.0	1.46	+41.1	1.27	−16.5
3.40		0.48		0.67		1.15		2.06		1.06	
2.68	− 6.7	1.08	+ 0.9	0.73	−35.6	1.34	+34.3	0.86	+37.2	1.00	− 9.0
2.50		1.09		0.47		1.80		1.18		0.91	
3.46	−11.3	0.76	−14.5	0.76	−21.1	1.07	+19.6	1.32	+18.9	1.22	−10.7
3.07		0.65		0.60		1.28		1.57		1.09	
3.75	−13.9	0.56	−21.4	0.78	−33.3	0.95	+46.3	1.81	+14.4	1.19	−10.1
3.23		0.44		0.52		1.39		2.07		1.07	
3.60	−10.3	0.77	− 9.1	0.78	−19.2	1.09	+33.9	1.37	+20.4	1.23	−12.2
3.23		0.70		0.63		1.46		1.65		1.08	

a marked reduction in all the subjects, in the order of severity in acute and chronic nephritis and malignant and benign essential hypertension. After the administration of depressants it increases to approach normal level only in benign essential hypertension, while in the other forms, it falls further below the normal in the order of malignant essential hypertension, acute and chronic nephritis.

Thus, it has been ascertained that in hypertensive diseases the protein fractions in plasma which show the most marked change are albumin and fibrinogen, the former being decreased and the latter increased. That these fractions are chiefly produced in the liver has been demonstrated by a series of investigations (Foster and Wipple,[1] Knutti,[2] Miller,[3] Yasuda,[4] and others) and it is known that functional disturbances in the liver are often accompanied by hypoalbuminemia and γ-globulinosis. Miller[3] and Ikushima[5] state that when in the blood the albumin is diminished, α-globulin first comes into compensatory increase, followed by β-globulin, fibrinogen and γ-globulin, and according to renin hypothesis, hypertensinogen, the precursor of hypertensin, is contained in α-globulin. Wuhrmann[6] infers that α-globulin contains initial antibodies, which are gradually transfered to γ-globulin, while Martin[7] states that γ-globulin is subjected to destruction in the liver under normal circumstance and in

TABLE

Plasma Protein Fractions of Nephritic Hypertension Before

Cases 1–5: chronic nephritis,

Case	Sex	Age	Depressants (mg.)	Blood pressure before & after depressants (mm.Hg)	Total protein g/dl	Total protein Change %	Albumin g/dl	Albumin Change %
1	♂	30	Apresoline (18)	b. 186–122 a. 164– 90	5.76 6.12	+ 6.3	1.94 2.01	+ 3.6
2	♂	56	Trapon (0.75)	b. 220–140 a. 182–126	8.57 7.65	−10.7	4.10 3.23	−21.2
3	♀	35	Apresoline (20)	b. 190– 84 a. 162– 74	7.72 7.72	± 0	4.32 4.36	+ 0.9
4	♂	29	C_6 (25)	b. 155– 90 a. 140– 90	7.35 7.16	− 2.6	3.37 3.14	− 6.8
5	♂	48	C_6 (25)	b. 175– 90 a. 160– 90	7.48 7.32	− 2.1	3.43 3.21	− 6.4
Average				b. 185–105 a. 162– 94	7.38 7.19	− 2.6	3.43 3.19	− 7.0
6	♂	28	C_6 (20)	b. 174– 90 a. 160– 74	7.20 7.04	− 2.2	3.34 3.07	− 8.0
7	♀	24	Apresoline (20)	b. 190– 90 a. 150– 50	6.28 6.02	− 4.1	2.59 2.53	− 2.3
Average				b. 182– 90 a. 155– 62	6.74 6.53	− 3.1	2.95 2.80	− 5.1

functional disorder of the liver this process is hampered, thus leading to the increase of γ-globulin in the blood. According to Shida[8] and Shioji,[9] the liver and kidneys act in collaboration in changing the blood proteins qualitatively, for the purpose of maintaining the colloidal osmotic pressure of blood at the normal height. Fibrinogen is said to be produced in the liver, (McMaster and Drury[10] and others), also in the bone marrows (Morawitz[11]) and it tends to increase compensatorily in hypoalbuminemia. Since, according to Berman,[12] Davis,[13] and Takahashi,[14] the hepatic blood flow is lessened to some extent in hypertensive diseases, it may be inferred that, in essential and nephritic hypertension, certain functional reduction of the liver due to the more or less extensive decrease of blood flow in it is followed by some decrease of the intrahepatic albumin production, which in turn causes hypoalbuminemia, and results in an accentuation of compensatory globulin production in the liver and the kidneys; in particular γ-globulin in the blood is markedly augmented through an extrahepatic hyperproduction, and abated intrahepatic destruction. It is also to mention that the production of fibrinogen in the bone marrows

IV

and After the Administration of Depressants

Cases 6–7: acute nephritis

α-globulin		β-globulin		Fibrinogen		γ-globulin		$\frac{A}{G}$	
g/dl	Change %	g/dl	Change %	g/dl	Change %	g/dl	Change %		Change %
0.45 0.43	− 4.4	1.07 1.30	+21.5	1.32 1.42	+ 7.6	0.97 1.07	+10.3	0.78 0.72	− 7.7
0.61 0.44	−27.9	0.85 0.52	−38.8	1.04 1.39	+33.7	1.98 2.07	+ 4.5	1.19 1.07	−10.1
0.83 0.82	− 1.2	0.94 1.05	+11.7	0.63 0.59	− 6.3	1.00 0.89	−11.0	1.56 1.58	+ 1.3
0.63 0.56	−11.1	0.99 0.99	± 0	1.06 1.17	+10.4	1.30 1.10	−15.4	1.15 1.18	+ 2.6
0.64 0.57	−10.9	1.01 1.02	+ 1.0	1.08 1.20	+11.1	1.32 1.13	−14.4	1.15 1.18	− 2.6
0.63 0.56	−11.1	0.97 0.98	+ 1.0	1.03 1.15	+11.7	1.31 1.25	− 4.6	1.17 1.15	− 1.7
0.46 0.58	+26.1	0.59 0.52	−11.9	1.02 1.16	+13.5	1.79 1.71	− 4.5	1.17 1.09	− 6.8
1.32 1.22	− 7.6	0.82 0.89	+ 8.5	0.50 0.44	−12.0	1.05 0.94	−10.5	0.81 0.83	+ 2.5
0.93 0.93	± 0	0.72 0.72	± 0	0.74 0.78	+ 5.4	1.37 1.30	− 5.1	0.98 0.95	− 3.1

is accentuated in compensation of the lowered albumin.

When the blood pressure is abruptly lowered by administration of various depressants, hexamethonium bromide (Methobromine), veratrum viride (Trapon), and 1-hydrazino-phthalazine (Apresoline), according to Freis,[15] Aizawa[16] and others, if the blood pressure reduction is appropriate, the blood flow in the main organs (liver, kidneys, heart, brain and others) is augmented in essential hypertension, especially in benign form, and in some nephritic hypertensions. In the present experiments on blood pressure lowering, as if to endorse those observations, in the benign essential hypertension, an increase of albumin and $\frac{A}{G}$ and a decrease of α- and β-globulins approaching normal values were ascertained as a sign of recovered function of the principal organs concerned with protein metabolism, due to the increase of the blood flow therein, while in the fibrinogen and γ-globulin, which are produced extrahepatically, no decrease could be demonstrated, rather showing a tendency to further increase. In malignant essential and nephritic hypertension, where the

pathologic changes of the blood vessels are advanced, though in some cases, if the progress of the disease is slight and lowering of blood pressure is appropriate, results of the same tendency as in benign form appeared, but in general, there occurred a higher reduction of the low albumin content and of the small $\frac{A}{G}$ ratio, suggesting further decrease of blood flow in various organs and a consequent reduction of functions, due to the blood pressure lowering, while the compensatory increase of globulin and fibrinogen was perceptible enough in malignant essential hypertension but not in the nephritic hypertension.

Thus, it may be inferred that, in evaluating the efficacy of blood pressure depressants, it is possible to guess the change in the blood flow, induced by the blood pressure lowering, in various organs, in particular, liver, kidneys and brain, by examining the change in plasma protein fractions before and after the application of the medicaments, and such a comparative examination may also give a clue to determining the progress of vascular diseases and classifying hypertensive diseases.

Summary

22 cases of essential hypertension (14 benign and 8 malignant), and 7 cases of nephritic hypertension (5 of chronic and 2 of acute glomerulonephritis) were injected intramuscularly with various blood pressure depressants, hexamethonium bromide (Methobromine), veratrum viride (Trapon) and 1-hydrazino-phthalazine (Apresoline), for effecting abrupt lowering of the blood pressure, and the protein fractions in the plasma before and after the blood pressure lowering were measured. The results are as follows:

1. The total plasma protein is slightly lower than the normal in all the hypertension forms, especially, in acute glomerulonephritis; upon lowering of blood pressure, the decrease is accentuated further in the hypertensions with disturbance of the renal function, being most marked in acute nephritis, followed by chronic nephritis and malignant essential hypertension. In benign essential hypertension, the value shows a very slight rise, if anything.

2. Albumin is reduced below the normal level in all forms of hypertension. Through lowering of the blood pressure, it is further diminished in the cases with renal damage, the reduction being marked in the order of malignant essential, chronic nephritic and acute nephritic hypertension. In benign essential hypertension, it tends to increase after the administration of the depressants.

3. The total globulin content is augmented beyond normal in all the forms, fibrinogen and β- and γ-globulins being especially in increase,

accordingly the ratio $\frac{A}{G}$ being always subnormal. Upon effected lowering of blood pressure, α- and β-globulins decrease slightly, apparently in essential hypertension. Fibrinogen tends more or less to an increase in all cases, particularly marked in malignant essential and nephritic hypertension. γ-globulin is found significantly increased in essential hypertension, especially in malignant form; on the contrary in renal hypertension, it tends rather to decrease. After the blood pressure lowering, the ratio $\frac{A}{G}$ increases towards normal value in benign essential hypertension, but is diminished further in all other forms.

4. It is mentionsworth that when the blood pressure is lowered immensely, even benign form may show changes similar to those in malignant and nephritic hypertension subjects.

5. No difference in results is observed by the use of different depressants.

6. An attempt is made to explain the pathologic physiology of the above presented findings. It is suggested that from the changes in the protein fraction picture of plasma certain functional disturbance of the important organs, such as kidney, brain, heart, liver and others, may be guessed and that some information on the evaluation of the blood pressure depressants can be obtained.

The expense of this investigation was partly defrayed from the Grant in Aid for the Medical Research, Medical Affair Bureau, Welfare Ministry.

References

1) Foster & Wipple, Am. J. Physiol., 1922, **58**, 407.
2) Knutti, J. Exp. Med., 1937, **65**, 455.
3) Miller, *ibid.*, 1954, **99**, 125.
4) Yasuda, Tohoku Journ. Exp. Med., 1937, **31**, 437.
5) Ikushima, Acta Haem. Jap. (Jap.), 1954, **17**, 59.
6) Wuhrmann, Schweiz. med. Wchr., 1952, 937.
7) Martin, Brit. J. Exp. Path., 1949, **30**, 231.
8) Shida, Tohoku Journ. Exp. Med., 1939, **35**, 304.
9) Shioji, *ibid.*, 1941, **40**, 336.
10) McMaster, Proc. Soc. Exper. Biol. & Med., 1928, **26**, 490.
11) Morawitz, Handb. d. Biol. d. Menschen, 1925, **4**, 96.
12) Berman, Arch. Surg., 1952, **65**, 430.
13) Davis, *ibid.*, 1951, **62**, 325.
14) Takahashi, J. Jap. Obst. & Gyn. Soc. (Jap.), 1953, **5**, 1055.
15) Freis, J. Clin. Invest., 1949, **28**, 353; Circulation, 1953, **8**, 199.
16) Aizawa, Saishin-igaku (Jap.), 1953, **8**, 10.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Experimental Formation of Gallstones by Means of Roundworm Eggs

By

Tokuo Atsumi

(熱海 徳夫)

From the Surgical Clinic of Prof. T. Maki, School of Medicine, Hirosaki University, Hirosaki

(Received for publication October 29, 1955)

Invasion of roundworms into the biliary tract is a routine matter of observation[1] in Japan, and it is well known that the cuticles of such invading roundworms become nuclei for the formation of gallstones, on which Sato[2] of our clinic has already published his report. The eggs left in the biliary tract by such worms also play a part in stone formation. Yoshida[3] for the first time reported that some gallstones he had observed microscopically were formed, making ascaris eggs their nuclei, in 1908. Yasuda[4] of our clinic chemically dissolved the nuclear parts of apparently ordinary pigment chalk stones and detected many ascaris eggs in a very high frequency. Figures of stone formation in the initial stage with pigment chalk precipitation on the shells of ascaris eggs were clinically obtained, by Maruyama[5] and Asakura[6] of our clinic. This series of findings suggests that the ascaris eggs show a deep significance as nuclei in pigment chalk stone formation in Japan. On the other hand, Asakura[7] has accomplished a crystallographic study of the ascaris egg shells and has published a very suggestive observation that the shells of fertilized ascaris eggs are, of all eggs of human parasites, the best adapted for forming nuclei of gallstones. In good agreement with his results, the author of this paper has succeeded in experimentally precipitating pigment chalk on ascaris eggs and further observing the mode and process of the precipitation, as reported in the following.

Experimental

Method

1) Ascaris eggs used: These were directly extracted from the uteri of female ascaris. The extracted mass contained fertilized and unfertilized eggs with and without albuminoid membrane and these were used in suspension in physiological saline.

2) Animals used: 38 male and 31 female mongrel Japanese dogs in normal health and 3–11 kg. in body weight were used. No special manipulation or restriction was given their feed, either before or after the operation, but the usual table leavings were fed throughout.

3) Grouping: In all the tests, 0.5–1.0 cc per kg body weight of 4% morphine hydrochloride solution was used for basal narcosis in combination with local anesthesia with 0.5% procaine hydrochloride. The animals were divided into the three following groups according to the experimental methods applied.

Group I. Ascaris eggs directly introduced into the gallbladder.

39 dogs were subjected to this method. Upon laparotomy, the gallbladder was punctured and the cystic duct was lightly ligated with silken threads, so that stasis was occasioned in the bladder without complete closure of the duct. Then, the ascaris egg suspension was introduced with a syringe directly into the gallbladder. 25 of the subjects had only ascaris eggs thus introduced, but the other 14 had 1.0 cc of calcium chloride injected in combination with the egg suspension.

Group II. Ascaris eggs introduced into the choledochus per duodenum. 30 dogs were used here. The egg suspension was introduced through a Nelaton's catheter No. 3 temporarily inserted transduodenally into the Vater's papilla. In this group, the cystic duct was left unligated in 15 dogs, half ligated, as in the above group, in 7 and in 8 cases it was completely ligated or cholecystectomy performed, for the purpose of observing the ascaris eggs in the biliary tract only in exclusion of the influence of the gallbladder.

Group III. Controls. With these the three following experiments were carried out: i. Foreign objects other than ascaris eggs were introduced into the gallbladder. These objects were sterilized objects, such as powdered charcoal, white sand grains, shreds of rubber tissue, each being about 1–3 mm^3 in size and 1.0 g. in total quantity. Each object was directly introduced into the gallbladder through an incision and the cystic duct was lightly ligated as in Group I above (10 dogs). ii. The ascaris egg suspension was merely introduced directly into the gallbladder, but no manipulation was applied to the cystic duct and the Vater's papilla (9 dogs). iii. In vitro: Samples of human gallbladder bile extracted at the time of surgical operations of the liver or the biliary tract diseases were centrifuged and sedimentated, and after attesting their freedom from eggs and cuticles of parasites, were mixed with ascaris egg suspension and kept in an incubator at 37°, for microscopic examinations at stated periods.

In all the above experiments, the bladdar bile was sampled before the experimental operations to assure that it was free from eggs of parasites and bile sand.

4) Appraisal of the results: After the lapse of the stated time,

the animals were operated upon for the second time, their bile in the gallbladder or the choledochus being extracted, and then the ascaris eggs in it were examined. In the case when pigment chalk, which seemed precipitation was found on them, the color, the form and the size of the granules were observed, their bilirubin reaction examined under a microscope under low magnification, using Kossa's method in the examination of the precipitate. In the fields where positive ascaris eggs were found, the frequency of the eggs with and without the precipitation was determined as classified by the sorts of the eggs. The properties of the bile, namely its pH value and its bacterial count were also examined at the same time.

Results

1. *Process of pigment chalk (calcium bilirubinate) precipitation on ascaris eggs*

Of all the 69 tested subjects, precipitation of pigment chalk granules on different kinds of ascaris eggs was observed in 14 cases or 20% in total. Under a microscope, these are observed as granules of dark brown color 3–5 μ in diameter—showing figures entirely identical with the pigment chalk granules found precipitated on ascaris eggs and cuticles in clinical cases. A test of these granules for bilirubin under the above mentioned method showed that they were rich in bilirubin. The precipitation showed some difference according to the sort of the eggs.

I. Fertilized eggs with albuminoid membrane

Precipitation was most conspicuous on these. In the early stage, the albuminoid membrane, the egg shells, and the egg cells were clearly observable, granules of calcium bilirubinate being sporadically precipitated on them. Figs. 1 and 2 show such eggs 4 days after their introduction into the gallbladder. In some cases, the precipitation become aggregated and dense as early as 7 days after the implantation, so that the egg shell becomes hidden. In about 3 weeks, the precipitation progresses further and the egg shells and the egg cells become indistinct (Fig. 3, 4). One month after the introduction of the eggs, the precipitated pigment chalk covers up the eggs in one aggregated mass and shows a figure of incipient gallstone (Fig. 5).

II. Fertilized eggs without albuminoid membrane

Precipitation of calcium bilirubinate was observed first on the 7th day (Fig. 6), and Fig. 7 shows such an egg on the 18th day. No such advanced precipitation as in the above was observed with these eggs.

III. Precipitation of calcium bilirubinate on egg cells

In fertilized but albuminoid membraneless eggs, it was often seen that the bile first penetrates into the egg cell and then the calcium bilirubinate precipi-

tation apparently has grown to cover the egg shell (Fig. 8).

Fig. 1 Fig. 2 Fig. 3 Fig. 4

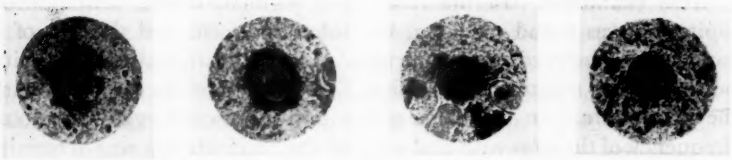


Fig. 1. The initial stage of pigment chalk precipitation on egg shell, 4 days after the insertion of fertilized ascaris eggs with albuminoid membrane into the gall bladder.

Fig. 2. The same as the above.

Fig. 3. Pigment chalk precipitation in the next stage, 18 days after the insertion.

Fig. 4. Another instance of the same, 18 days after insertion.

Fig. 5 Fig. 6 Fig. 7 Fig. 8

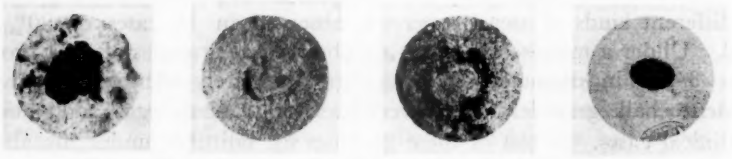


Fig. 5. One of the eggs, one month after insertion.

Fig. 6. One instance of precipitation, 7 days after the insertion of fertilized ascaris eggs without albuminoid membrane into the gall bladder.

Fig. 7. The same, showing aggregation of pigment chalk granules around the egg. A picture taken 10 days after the insertion.

Fig. 8. Pigment chalk precipitation in egg cells, 8 days after the insertion of fertilized membraneless eggs into the gall bladder.

IV. Unfertilized eggs

Only in one instance of unfertilized eggs with albuminoid membrane was observed a slight precipitation. No precipitation was ever observed on unfertilized eggs without albuminoid membrane.

2. *Results by groups*

I. Group I: Precipitation was observed in 7 or 17.9% of the 39 animals of this group. Of the 25 animals inoculated only with ascaris egg suspension, 2 cases, and of the 14 animals inoculated with calcium chloride solution in combination with the suspension, 5 cases showed precipitation of calcium bilirubinate on the eggs.

II. Group II: Precipitation was observed in 7 or 23.3% of the 30 animals. Positive results were obtained in 3 of the 15 dogs with their cystic ducts left unligated and in 3 of the 7 dogs with their cystic ducts lightly ligated.

In 1 case among these positive cases, the precipitation was found on ascaris eggs in the choledochus. In the subgroups where the gallbladder was extirpated or the cystic duct was completely closed, precipitation was observed on one case only on eggs in the choledochus.

III. Control experiments: a. When foreign objects other than ascaris eggs were used, no accretion of the foreign matter or precipitation of calcium bilirubinate on them was observed.

b. When merely ascaris eggs were introduced directly into the gallbladder, without giving any manipulation to the cystic duct or the region of the Vater's papilla, no precipitation of calcium bilirubinate was ever observed either on eggs in the biliary ducts or on those in the gallbladder.

c. In vitro experiments, precipitation was observed in 4 cases among 10. The bile used in these 4 positive tests was taken from 4 cases, one from cases of ascariasis of biliary tract, and another from liver abscess cases, and the others from cases of cholecystitis. All the eggs on which such a precipitation was observed were fertilized eggs. The earliest precipitation in vitro occurred one month after the addition of bile.

3. *Relation between sorts of ascaris eggs and frequency of precipitation*

In all the above experiments, fertilized eggs with albuminoid membrane showed the highest percentage of precipitation (68.5%), followed in percentage by fertilized albuminoid membraneless eggs (29.9%) and unfertilized eggs with albuminoid membrane (1.5%), no instance of precipitation on unfertilized membraneless eggs being observed.

4. *Relation between precipitation of calcium bilirubinate and bile stasis*

There was no marked difference in the frequency of precipitation observed in Groups I and II, but in the Group II, the frequency was larger in the animals with their cyctic ducts lightly ligated (3 positive cases among 7) than in those with their cystic ducts left intact (3 positive cases among 15), while with the animals not operated upon to cause stasis of their bile in the Group III, no instance of precipitation was ever observed.

5. *Properties of bile*

I. pH values: Of the 69 animals in the Groups I and II above, the pH values of their bile prior to and following the operation were determined with 44 cases. In the majority of the cases (33 among 44), the pH values of their bile showed a tendency to acidification. The details by groups and occurrence of calcium bilirubinate precipitation were as follows:

a. In Group I, the pH values tended to acidity in 5 of the 7 cases that showed precipitation and in 12 of the 19 cases that showed negative results whose change in pH values of bile was clearly observed.

b. In Group II, all the cases of which the change in pH values were followed up showed a turn to fall, except two cases, one from among the 6 cases with positive and the other from among 12 cases with negative results in the precipitation tests.

c. The change in pH was recorded with 8 cases of foreign matter inclusion tests in the control experiments, of which 6 cases showed acidification of the bile.

II. Bacteria in the bile : In Groups I and II, the bacterial counts were determined by cultures using bile from the gallbladder and the biliary ducts taken before and after the operation performed on 53 cases, including the cases in Group II where the ascaris eggs in the biliary ducts were examined, of which the counts were taken only of the duct bile. The detected bacilli chiefly consisted of colibacillus, no other kinds of bacilli being observed except some staphylococci albi found in a few cases only. Among the 14 cases where calcium bilirubinate precipitation was demonstrated, bacteria in duct bile were detected in 11 cases, of which colibacilli were detected in 4 of the 5 tested cases in Group I and in 5 of the 6 tested cases in Group II. Staphylococci albi were detected only in one case of the dogs inoculated with ascaris egg suspension only in Group I, and in this case also colibacilli were found in mixed infection. No marked difference was found in the frequency of detection of colibacillus between the cases with positive (81.8%) and negative (75.5%) calcium bilirubinate precipitation.

Summary and Discussion

As detailed in the above, the author made the experiment of introducing ascaris eggs into the gallbladder or into the biliary ducts of dogs and causing stasis of bile in them, and detected precipitation of calcium bilirubinate in 20% of the total tested cases. It is generally considered[8] that in dog the experimental formation of gallstones is very difficult, so the above experiment showing a high percentage of successful cases is worthy of our attention.

As reliable reports demonstrating ascaris eggs detected in gallstones, we can point to those by Yamamoto,[9] Nomamoto[10] and Nishimura,[11] besides that by Yoshida[3] cited above, but as to experimental studies on the relation between eggs of parasites and gallstone formation, we can count only those reported by Inoue[12] and Kono[13] who have taken the part played by the eggs of clonorchis sinensis in gallstone formation in the liver as the postulate of their studies. Hepatic clonorchiasis is rather

frequent in Japan, but the part played by ascaris lumbricoides is far more important in respect to surgical diseases of the biliary tract. In the above experiment I could show that the eggs of ascaris lumbricoides like its cuticles have a close connection with the formation of pigment chalk stones which is characteristic of gallstone in Japan.

It must be noted that precipitation of calcium bilirubinate is more apt to be incurred by fertilized eggs of ascaris. This is, in the first place, due to the higher crystallographic stability of the structure of the shell of a fertilized egg than that of an unfertilized egg, and the consequent better adaptation of the former as a nucleus of a gallstone. In the second place, the albuminoid membrane also has, as Asakura states, a great significance for the precipitation of calcium compound, when it putrefies and calcium carbonate deposit takes place on it. The utterly negative results of the experiments with foreign objects other than ascaris eggs may be taken to endorse the high suitableness of ascaris eggs as nuclei of pigment chalk stones. The control experiments with ascaris eggs introduced in the gallbladder, no stasis of bile being induced in it, produced scarcely any case showing stony precipitation, indicating the strong influence of bile stasis on stone formation. Fukushima[14] of our clinic has proved experimentally that an invasion of an ascaris into the common bile duct causes a strong contracture of the Oddi's muscle by mechanically stimulating the Vater's papilla with the accompanying bile stasis and dilatation of the biliary ducts. This fact is in agreement with the rather frequent occurrence of precipitation in Group II than in Group I above.

It must be also considered that in these experiments, the dogs used were fed with the usual left-over rice and no manipulation or restriction was placed on their feed. In the past, we have heard discussions on the influence of diet on gallstone formation, but the above experiments show that even if the influence of the general bodily conditions does not come into consideration, if only the local requirements are fulfilled, precipitation of calcium bilirubinate can take place. This precipitation may take place as much in the gallbladder as in the biliary ducts, the only difference lying in the time when the precipitation begins—4–5 days in the former and 7–10 days in the latter at the earliest, after the entrance of the ascaris eggs.

Taking the above experimental results into consideration, we may conclude that, in ascariasis of the biliary tract, when ascaris eggs are laid in the tract, a foundation for a very likely and prompt formation of gallstones is laid. We may also find some explanation in this for the frequency of gallstones being found in the biliary ducts and the liver in Japan.

Next, I will speak a word on the properties of the bile.

According to Oba,[15] the pH value of normal dogs ranges between

6.48 and 7.52. In the above experiments, the value stood between 6.41 and 7.34, but after the operation for the introduction of ascaris eggs, it tended to decrease to the acid side. It has been pointed out by many researchers that a tendency to acidification of bile causes precipitation or coagulating out of its components and thus becomes an important factor in gallstone formation. In the above experiments, the acidification of the bile must have also played an outstanding part in gallstone formation.

The rate of bacilli detection in the bile of cholelithiasis cases is generally higher in Japan than in Western countries, colibacillus occupying the leading position among the detected bacilli, presumably by ascendant infection. In the above experiments, there was no perceptible difference in the frequency of bacilli detection between the bile in which precipitation of calcium bilirubinate on ascaris eggs was observed and that wherein no precipitation was found (81.8%: 75.7%). This suggests that the colibacillus infection, rather than playing a directly important part in stone formation, exerts a secondary influence on the composition of the bile through inducing inflammatory changes in the adjacent tissues, and this influence results in materializing conditions favorable for stone formation. As bilirubin must combine with calcium before precipitating, much calcium is required for pigment chalk stone formation. In Group I above precipitation was slightly more frequent in the animals injected with calcium chloride solution in combination with ascaris egg suspension than in the other animals. Now, it has been demonstrated by Hashimoto[16] of our clinic that the calcium content in the bile is increased in the initial stage of inflammation or stasis of the gallbladder.

Conclusion

As a link in the series of experimental studies concerning gallstone formation due to roundworm, the following experiments were conducted with dogs as subjects. Ascaris eggs were introduced into the gallbladder and the biliary ducts of dogs, the consequent stasis induced in them by the application of various methods, and then the eggs were taken out by reoperation at stated intervals. Finally the eggs and the composition of bile were examined. The results obtained were as follows:

1. Though no manipulation or restriction was applied to the feed of the dogs, precipitation of calcium bilirubinate on the ascaris eggs was observed on 20% of the total cases thus examined.

2. In most of the experimented animals, infection by colibacillus was observed in the biliary tract, but the most important factor causing the precipitation of calcium bilirubinate in these experiments was presumable the stasis of bile.

3. Of the different conditions of the eggs, fertilized eggs were more frequently subject to precipitation of calcium bilurubinate than unfertilized eggs, and among the former, those with albuminoid membranes were most apt to receive precipitation on them.

4. Of the different groups of the experimental animals, the group injected with calcium chloride solution in combination with the ascaris egg suspension and with their cystic ducts lightly ligated showed the highest frequency of precipitation.

5. In general, in the early stage following the introduction of ascaris eggs, calcium bilirubinate is found to have precipitated in sporadic dots on the albuminoid membranes and the shells of the eggs, so that these and the egg cells are still clearly visible, but in 3 weeks, the precipitation will gain in scope and density, so that the structure of the eggs will be blurred. The time required for the initiation of the precipitation after the introduction of the eggs was 4–5 days in the gallbladder and 7–10 days in the biliary ducts, while it took 30 days for the first precipitation to occur in vitro.

6. In some cases of fertilized but albuminoid membraneless eggs, calcium bilirubinate seemed first to seep into the egg cell before precipitation on the egg shell, and to grow thence toward the shell.

7. From the above results of the experiments, I am led to the conclusion that, in ascariasis of the biliary tract, when ascaris eggs are laid or left in the biliary tract, a groundwork has already been laid for a rapid formation of pigment chalk stones in a very high probability.

Thanks are due to the Education Ministry for the grant from the Scientific Research Fund to this study. T. Maki.

References

1) Maki, Tokyo Iji Shinshi (Jap.), 1952, **69**, 6.
2) Sato, Hirosaki Igaku Zasshi (Jap.), 1955, **6**, 194.
3) Yoshida, Tokyo Iji Shinshi (Jap.), 1908, **1551**, 365.
4) Yasuda, Hirosaki Igaku Zasshi (Jap.), 1955, **6**, 391.
5) Maruyama, *ibid.*, 1954, **5**, 97.
6) Asakura, *ibid.*, 1954, **5**, 102.
7) Asakura, Tohoku Jour. exp. Med., 1956, **64**, 105, 117.
8) Miyake, Saishin Igaku (Jap.), 1948, **3**, 456.
9) Yamamoto & Sato, Nippon Geka Gakkai Zasshi (Jap.), 1917, **18**, 192.
10) Nomamoto, Igaku Kenkyu (Jap.), 1944, **18**, 415.
11) Nishimura, Rinsho Geka (Jap.), 1948, **3**, 145.
12) Inoue & Ikeda, Jikken Shokaki Byogaku (Jap.), 1938, **13**, 814.
13) Kono, *ibid.*, 1942, **17**, 420.
14) Fukushima, Hirosaki Igaku Zasshi (Jap.), 1955, **6**, 58.

15) Oba, Okayama Igakkai Zasshi (Jap.), 1933, **45**, 1.
16) Hashimoto, Hirosaki Igaku Zasshi (Jap.) 1956, **7**, 323.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Biochemical Studies on Carbohydrates

CLXXXV. Comparison of Group A and O Substances from Pig Stomach Mucus

2nd Report (A Supplementary Note to the 1st Report)

By

Zensaku Yosizawa

(吉沢 善作)

From the Medico-chemical Institute, Tohoku University, Sendai; Director: Prof. H. Masamune

(Received for publication, October 30, 1955)

The writer informed in 1954[1] that Group A and O substances separated by alkaline treatment at 0–3°C of pig stomach mucus showed distinct discrepancy from each other in L-fucose content and optical rotation but not in other chemical and physical respects. In that work hexosamines were estimated altogether. Afterwards, however, differences among the glucosamine:glactosamine ratios of group mucopolysaccharides belonging to different blood groups were mentioned by Leskowitz and Kabat[2] and by Rondle and Morgan.[3] According to the former group of authors, the average glucosamine:galactosamine ratios in human Group A, O and B substances and pig Group A and O substances are 1.6, 2.5, 2.8, 1.5 and 2.2 respectively although the ratio varies widely in horse and bull group substances irrespective of the group specificity. The latter group of authors examined the group mucopolysaccharides from ovarian cyst fluids, and found the ratio to be 0.7–1.6 in the A substances, 1.7–3.5 in the B substances, 3.2 in a Le^a substance and 2.6–12.7 in the H substances. Furthermore, regarding similar preparations from ovarian cyst fluids, Gibbons and others[4] recorded the average galactose:hexoxamine ratio of A substances of about 0.6, that of H substances of about 1.2 and the ratio of a B substance of 1.5, and also stated that the L-fucose:hexosamine ratio is generally lower in A substances than in H substances, and the B substance lies between A and H substances in this respect, L-fucose contents being mostly larger in H substances than in A and B substances. Sialic acid content was found to decrease in the order B, A and H from left to right.

To scrutinize those relations in the group substances obtainable from pig stomach mucus by his procedure, the writer applied the methods for determination of individual hexosamines and Molisch positive mono-

saccharides recently devised or improved in this Institute, with results that 1) the glucosamine:galactosamine ratios in the Goup A, O and AO substances newly prepared were 1.8, 4.5 and 2.1 respectively, in accord in trend with the results of the American and English investigators above, 2) the galactose:hexosamine ratio and L-fucose:hexosamine ratio in the preparations did not differ noticeably from group to group although they showed some tendency of variation which Gibbons *et al.*[4] had mentioned, and 3) the Group O substance almost lacked sialic acid contrary to the Group A and AO substances, suggesting absence of this acid in the pure Group O mucopolysaccharide from the present source. To add, when the preparations are compared with one another with respect to the analytical figures of hexosamines, L-fucose and sialic acid and optical rotation, the AO substance, which was similar in composition to a preparation from the mucus pooled without discrimination between A and O potencies, is regarded to be composed of a great part of pure Group A mucopolysaccharide and a small part of pure Group O mucopolysaccharide.[5]

Experimental

Preparation of the Group A, O and AO substances

The procedure in a foregoing article of the writer[1] was applied, employing pooled Group A (Group A and O(E) active at dilutions of $1:>10^5$ and $1:<10^3$ respectively), O (Group A and O(E) active at dilutions of $1:<5\times10^2$ and $1:>10^4$ respectively) and AO (Group A and O(E) active at dilutions of $1:>10^5$ and $1:>4\times10^3$ respectively) mucus as materials. The preparations were all biuret-negative, and Molisch and indirect- and direct-Osaki-Turumi positive.

Properties of the preparations

Group activity. The Group A preparation (Ch A) was Group A active at a dilution of $1:8\times10^7$, but Group O active at dilutions of $1:2\times10^3$ (against anti-OS saliva chicken serum) and $1:10^3$ (against an eel serum) and the corresponding group potencies of the Group O preparation (Ch O) were $\times5\times10^2$, $\times8\times10^4$ and $\times4\times10^4$, as expressed in dilutions. The AO preparation (Ch AO) showed A potency at a dilution of $1:2\times10^7$ and O potency at dilutions of $1:8\times10^5$ (against an anti-OS saliva chicken serum) and $1:2\times10^5$ (against an eel serum). The antisera were diluted to titre 16 before use.

Optical rotation in water.

$$\text{Ch A} \quad [\alpha]_D^{15}=\frac{-6.20\times100}{0.8\times1}=-25.0^\circ$$

$$\text{Ch O} \quad [\alpha]_D^{15}=\frac{-0.43\times100}{1.1\times1}=-39.1^\circ$$

Ch AO $[\alpha]_D^{15}=\frac{-0.26\times 100}{0.9\times 1}=-28.9°$

Paper partition chromatography of the sugar components. It was processed as had been also reported,[1] and all the preparations proved to contain glucosamine, galactosamine, galactose and L-fucose.

Quantitative composition. See Table I.

TABLE I

Composition of the Group A (Ch A), O (Ch O) and AO (Ch AO) Preparations

A. Sugar Contents

Analysis	Ch A		Ch O		Ch AO	
	In per cent	In equivalents per equivalent weight	In per cent	In equivalents per equivalent weight	In per cent	In equivalents per equivalent weight
N*	4.6		4.5		4.2	
Glucosamine†	22.6	0.86	29.6	0.99	24.2	0.90
Galactosamine†	12.6	0.46	6.6	0.22	11.5	0.43
Total hexosamines	35.2	1.33	36.2	1.21	35.7	1.32
Galactose‡	26.5	1.0	30.0	1.0	27.1	1.0
L-Fucose§	10.3	0.43	12.8	0.46	11.0	0.45
Sialic acid//	3.1	0.06	0.5	0.01	3.4	0.06
Ash¶	0.8		1.0		0.6	

* Micro Kjeldahl. † Masamune and Yosizawa[6]. ‡ Masamune and Sakamoto[7], § Masamune and Sakamoto[8], // Werner and Odin (With orcinol and by the direct Ehrlich reaction)[9], ¶ Pregl method without use of H_2SO_4.

B. Ratios between sugars

Substance	Glucosamine / Galactosamine	L-Fucose / Hexosamine*	Galactose / Hexosamine*	L-Fucose / Galactose	Sialic acid / Galactose
Ch A	1.79	0.29	0.75	0.39	0.12
Ch O	4.48	0.35	0.83	0.43	0.02
Ch AO	2.10	0.31	0.76	0.41	0.13

* Signifies total amount of the hexosamines.

Through the Grant Committee for Scientific Researches, the Ministry of Education gave a grant in aid to us. H. Masamune.

References

1) Yosizawa, Tohoku J. Exp. Med., 1954, **61**, 45.
2) Leskowitz & Kabat, J. Am. Chem. Soc., 1954, **76**, 4887.

3) Rondle & Morgan, Bioch. J., 1955, **59**, xiii.
4) Gibbons, R. A., Morgan & Gibbons, M., *ibid.*, 1955, **60**, 428.
5) Cf. Numabe, Tohoku J. Exp. Med., in press.
6) Masamune & Yosizawa, *ibid.*, this volume, p. 53.
7) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
8) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.
9) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 231.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Biochemical Studies on Carbohydrates

CLXXXVI. Oligosaccharides Separated after Acetolysis of the Group Mucopolysaccharide from Pig Gastric Mucus

First Report: "Gastro-N-trisaccharide"*

By

Hajime Masamune, Zensaku Yosizawa and **Mituo Haga**
(正宗 一) (吉澤善作) (芳賀光男)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, October 30, 1955)

The group mucopolysaccharide prepared by alkaline scission at room temperature of pig stomach mucus was subjected to acetolysis, and after subsequent deacetylation, fractioned by means of a cellulose column. And, from the 49th–90th of the 90 fractions a trisaccharide composed of acetylglucosamine, galactose and acetylgalactosamine was obtained in crystalline form. Treatment of it with hypoiodite changed the glucosamine into glucosaminic acid, indicating that the acetylglucosamine residue stands at its reducing terminal. Since the trisaccharide was Morgan-Elson negative and gave 0.98 mole of formaldehyde per mole when oxidized with sodium metaperiodate, the terminal acetylglucosamine must be bound at its 4-position by the adjacent hexose unit (Cf. Kuhn *et al.*[1]). In this oxidation, formic acid formed scarcely and the galactose residue was attacked very slowly. Therefore, the galactose residue lies intermediately in the molecule and its two neighbouring glycol-OH groups oxidized take trans-positions to each other (Cf. Courtois *et al.*[2]), in other words, galactose is assumed as bound at its 4-position glycosidically by the acetylgalactosamine residue which stands at the non-reducing terminal. Finally, the trisaccharide consumed 3.2 moles of the metaperiodate per mole before the galactose residue was nearly entirely broken up. In short, the trisaccharide must be an acetylglucosamine-4-galactosido (1,5)-4-acetylgalactosaminide (1,5). It is named *gastro-N-trisaccharide.*

Experimental

The Group Mucopolysaccharide as the Material

It was prepared as described by Yosizawa,[3] namely, fresh pig stomach

* Read before the 28th General Meeting of the Japanese Biochemical Society, Tokyo, Nov. 1-3, 1955.

mucus was digested with 2 volumes of 1.5 *N* NaOH for 24 hours at room temperature, and the acid-soluble fraction was deproteinized. The product was biuret negative, and Molisch- and indirect and direct Osaki-Turumi-positive. N 4.2%, glucosamine (Masamune and Yosizawa[4]) 24.3%, galactosamine (Masamune and Yosizawa[4]) 11.5%, galactose (Masamune and Sakamoto[5]) 26.8%, L-fucose (Masamune and Sakamoto[6]) 12.0%, sialic acid (direct-Ehrlich method of Werner and Odin[7]) calcd. as $C_{11}H_{19}NO_{10}$ 5.0%, ash (Pregl without use of H_2SO_4) 1.1%. The assay according to Masamune *et al.* (glass plate method)[8] showed it to be Group A active at a dilution of $1:4\times10^6$, and Group O active at dilutions of $1:1\times10^5$ and of $1:5\times10^4$ against an anti-O saliva chicken- and an eel-serum respectively. And, it gave a single boundary after electrophoresis in an alkaline and an acidic buffer, as shown in Fig. 1.

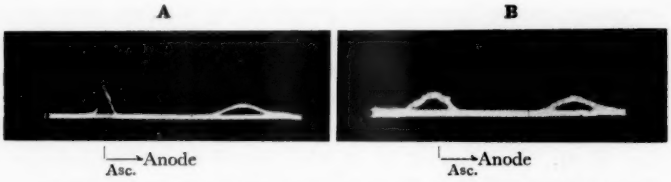


Fig. 1. Showing the electrophoretic patterns (4/5×) of 0.5% solutions of the group mucopolysaccharide in phosphate buffer pH 7.8, I 0.145 (**A**) and acetate buffer pH 4.6, I 0.2 (**B**). Temperature of test and current were 10°C and 8 mA respectively in (**A**) and 11°C and 7 mA respectively in (**B**). Exposure 60 minutes after starting current.

Preparation of Gastro-N-trisaccharide

i) Acetolysis. A 20 g. lot of the material was slowly added to a mixture of 150 cc. of acetic anhydride and 10 cc. of conc. sulfuric acid under cooling in a freezing mixture, and stood in a sealed tube for 5 hours at room temperature (20–22°C), for 12 hours at 37°C and for 10 hours at 50–52°C in succession, with occasional shaking. The mixture was then cooled in ice, and the insoluble part was separated on a glass-filter with suction and washed with 20 cc. of acetic anhydride. The filtrate and washing were united, poured into 1 *l.* of ice-chilled water containing 150 g. of sodium acetate (crystalline) under vigorous agitation, and after neutralization with sodium carbonate, exhausted with changes of chloroform. The chloroformic solutions were washed with water in a separatory funnel (The fluids mixture separated difficultly into two layers so that NaCl was added) and dried with dry $CaCl_2$. It was distilled in vacuo, and redistilled three times with abs. ethanol. The final still-residue was dried in vacuo over H_2SO_4. Yield, 17.8 g.

ii) Deacetylation. It was deacetylated according to Kuhn and Kirschenlohr[9] as follows. The substance was dissolved in 700 cc. of methanol saturated with ammonia at 0°C, stood for 6 hours at 20–22°C, shaking occasionally, and distilled under reduced pressure. The still-residue was further distilled twice with 50 cc. of abs. methanol and dried. It was next taken up in 100 cc. of water. A small insoluble part was centrifuged off and washed with 20 cc. of water. The supernatant and washing were passed together through a 2.5×30 cm. column of Dowex 50 (H-form) and a 3.5×30 cm. column of Dowex 1 in turn. The columns were subsequently washed with water, until no Molisch-positive substances came out any more (About 2 *l.* of water were required). The effluent and washing were distilled at below 40°C to syrupy consistency, repeatedly distilled with 50 cc. portions of abs. ethanol and dried in a vacuum desiccator (H_2SO_4). 10.5 g. of a powder were given.

iii) Chromatography. It was taken up in 80 cc. of a mixture of butanol, pyridine and water in the proportions of 5:3:2 by volume and poured onto a 5×50 cm. column of a cellulose powder wetted with the solvent above. Chromatography was effected by continuous dropping of the same solvent. The first 500 cc. portion of the effluent was discarded and successive ninety 30 cc. fractions were collected separately. Each of the fractions was added to with 30 cc. of water and 70 cc. of ether and shaken in a separatory funnel, and the ethereal layer was separated after standing the mixture and washed with 20 cc. of water. The watery layer and washing were distilled in vacuo to 1–2 cc. Every fifth condensate was paper-chromatographed with the mixed solvent above by the technique of Masamune and Yosizawa[10] and the spots were indicated with aniline hydrogen phthalate. Frs. 49–90, which were found to contain chiefly a sugar of an Rf value somewhat higher than that of lactose, were united and distilled under reduced pressure. And the still-residue was freed perfectly from water by several redistillations with 50 cc. of abs. ethanol each. It was followingly exhausted with hot abs. methanol and the insoluble part was taken up in 10 cc. of water (A small insoluble part was removed) and condensed to 1–2 cc., followed by addition of abs. ethanol in small portions until slight opalescence. On standing the solution, crystals in needle form appeared. Additional 80 cc. ethanol was added in portions, and the mixture was stood overnight. The crystalline deposit was collected on a suction funnel, and washed with 20 cc. of abs. ethanol and 5 cc. of ether. Yield 56 mg. The substance was recrystallized several times. It melted at 273–275°C (decomp.). When chromatographed with the solvent above (butanol-pyridine-water (5:3:2 by volume)) at 24–26°C, it moved with an Rr (rate of flow as compared with rhamnose) value of 0.37.

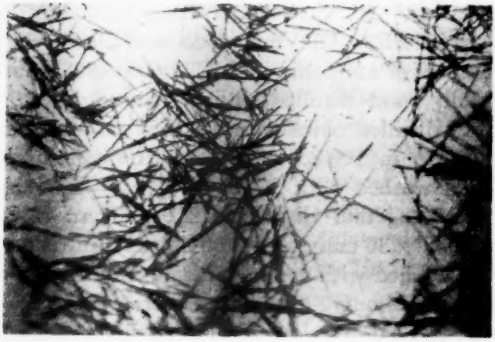


Fig. 2. Microphotograph of crystals of gastro-N-trisaccharide.

The whole procedure of preparation was repeated with new lots of the material.

Properties of Gastro-N-trisaccharide

Test-tube tests. It gave the positive Molisch reaction but not Morgan-Eison reaction.

Paper chromatography. 5 mg. of substance were heated with 2 cc. of 0.5 *N* HCl in an ampoule at 100°C for 10 hours, and the hydrolysate was freed from the hydrochloric acid as far as possible by evaporation at 30-40°C in vacuo over solid $CaCl_2$ and NaOH, adding water from time to time. Finally it was dried completely. The residue was dissolved in 0.1 cc. of water, and 0.02 cc. of the solution was sampled on a 12×55 cm. sliver of Toyo Roshi filter paper no. 3 to develop a chromatogram by descending irrigation with solvent butanol-pyridine-water (5:3:2 by volume). Spots of glucosamine, galactosamine and galactose were indicated by aniline hydrogen phthalate (Fig. 3).

Quantitative analysis. No decrease of weight occurred, when the substance was heated in vacuo at 105°C over P_2O_5 for 5 hours. The analytical figures are embodied in Table I.

The molecular weight is calculated at 596 from the iodine use (Theory 586).

Optical rotation in water.

$$[\alpha]_D^{17} = \frac{+1.32 \times 100}{1 \times 0.94} = +140.4^\circ \text{ (no mutarotation)}$$

Infrared spectrum (The writers are indebted to Mr. Y. Ikegami in the Chemical Research Institute of Non-aqueous Solutions). Measurement of the infrared absorptions was conducted in a Perkin-Elmer Model 21 recording infrared spectrophotometer with a NaCl prism by KBr disk

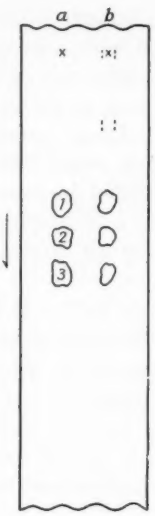


Fig. 3. Chromatogram of the hydrolysate of gastro-N-trisaccharide. Temperature and duration of test 24–26°C and 16 hrs. respectively. Indicator the aniline hydrogen phthalate reagent of Partridge. The reference run was carried out using 0.01 cc. of a 2% solution with respect to individual sugars.

a : reference run, b : main run. 1 : galactosamine, 2 : glucosamine, 3 : galactose.

TABLE I

Composition of Gastro-N-trisaccharide

Figures express percentages.

Analysis	Found	Calcd. for $C_{22}H_{38}O_{16}N_2$
N*	4.70	4.78
C†	45.43	45.05
H†	6.51	6.48
Glucosamine‡	29.9	30.5
Galactosamine‡	29.9	30.5
Galactose§	29.6	30.7
Acetyl//	14.3	14.7
Iodine use as glucose¶	30.2	30.7
Ash**	0.0	0.0

* Micro Kjeldahl. † Pregl ; Kindly analyzed by Mr. M. Suzuki in the Chemical Department. ‡ Masamune and Yosizawa.[4] § Masamune and Sakamoto[5]. // Suzuki[11]. ¶ Macleod and Robison[12]. ** Pregl.

method. The spectrum given is shown in Fig. 4.

Experiments Regarding Structure of Gastro-N-trisaccharide

Oxidation with NaOI and examination of the product thereby. i) 10 mg. of the substance were dissolved in 10 cc. of water, added to with 7 cc. of 0.02 *N* I_2, and after alkalization with 0.5 cc. of 5% Na_2CO_3, stood at 20–21°C for 1 hour in a dark room. The reaction mixture was acidified with 1 cc. of 2 *N* HCl and distilled

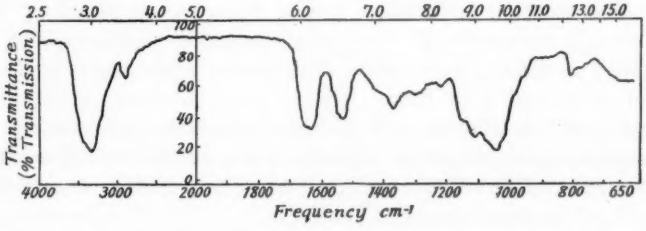


Fig. 4. Infrared spectrum of gastro-N-trisaccharide.

under reduced pressure with 99% ethanol to a small volume repeatedly. The final condensate was distilled to dryness, washed with a mixture of 5 cc. of abs. ethanol and 10 cc. of ether many times until the substance was perfectly decolorized, and dried. The powder was taken up in 10 cc. of water and desalted by passing through a 1×15 cm. column of Dowex 50 (H-form), which was followingly washed with 300 cc. of water. The effluent and washing were united, distilled in vacuo to a small volume and made up to 5 cc. with water in a measuring flask. This solution was subjected to paper-chromatography (ii) and analysis (iii).

ii) 1.5 cc. of it were added to with 1.5 cc. of 1 *N* HCl and heated in a sealed tube at 100°C for 10 hours. When cold, the hydrolysate was freed from most of the hydrochloric acid like the corresponding hydrolysate of the trisaccharide above. The last dry residue was dissolved in 0.07 cc. of water, and chromatograms of two 0.02 cc. portions were developed on 12×55 cm. paper slivers by descending irrigation with solvent butanol-pyridine-water (5:3:2 by volume). Glucosaminic acid, galactosamine and galactose were detected (Fig. 5).

iii) Analytical figures of the solution expressed in per cent of the original trisaccharide are tabulated as follows.

Analysis	Found	Calcd. for $C_{22}H_{38}O_{17}N_2$
Glucosamine*	<1	0
Galactosamine*	29.1	29.7
Galactose†	29.0	29.9

(* Masamune and Yosizawa[4], † Masamune and Sakamoto.[5])

Oxidation with $NaIO_4$ and examination of the products thereby. i) 29.3 mg. of the trisaccharide were dissolved in 15 cc. of 0.1 *M* acetate buffer of pH 5.0 and after addition of 5 cc. of 0.32 *M* $NaIO_4$ solution, stood at 15°C. 1 or 2 cc. portions were pipetted out at intervals to estimate the amounts of the oxidant remaining unreduced and the galactose residue unoxidized and those of formic acid and formaldehyde produced.

ii) Consumption of $NaIO_4$. It was measured as described by Masamune and Yosizawa.[13] To 2 cc. of the reaction mixture above, 10 cc. of 8% (w/v) $NaHCO_3$, 4 cc. of 0.1 *N* arsenious oxide and 0.5 cc. of 20% (w/v) KI were added in turn. The mixture here was stood at room temperature for 15 minutes and titrated with 0.02 *N* I_2, taking a 1% starch solution as an indicator. The blank was carried out by substituting water for the reaction mixture in i). 3.2 moles of $NaIO_4$ were consumed for a mole of the trisaccharide (Table II).

iii) Formation of formic acid. Carlquist's method[14] was employed.

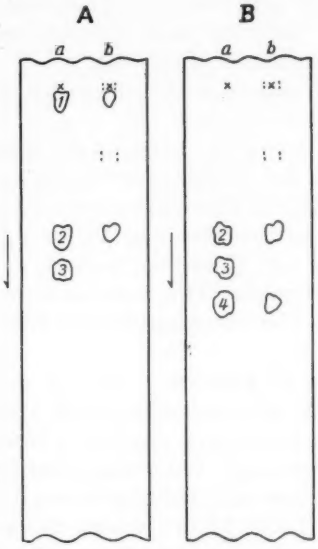


Fig. 5. Chromatograms of the hydrolysate of gastro-N-trisaccharide treated with sodium hypoiodite. Temperature and duration of test 24–26°C and 16 hrs. respectively. Indicators: ninhydrin (in **A**) and aniline hydrogen phthalate (in **B**). The standard solution for reference run contained 2% glucosaminic acid and individual sugars.

a: reference run, b: main run. 1: glucosaminic acid, 2: galactosamine, 3: glucosamine, 4: galactose.

TABLE II

The Course of Oxidation of Gastro-N-trisaccharide with $NaIO_4$

Time of oxidation (hrs.)	$NaIO_4$ consumption (mole/mole)	HCOOH production (mole/mole)	HCHO production (mole/mole)	Galactose remaining unoxidized in per cent of the trisaccharide
6	1.6			
24	2.1	0	0.77	18.9
45	2.6			10.2
72	3.2	0.2	0.98	0.5

2 cc. of the reaction mixture in i) and 5 cc. of water were mixed together, and after addition of 0.2 cc. of ethylene glycol, stood at room temperature for 20 minutes until excess $NaIO_4$ was completely destroyed. Then, formic acid was titrated with 0.01 *N* NaOH. Indicator 1% phenolphthalein in ethanol. The blank was processed with a mixture of the $NaIO_4$ solution,

which had been treated with ethylene glycol, and the substrate solution in the acetate buffer. Only 0.2 mole of formic acid formed in 72 hours for a mole of the trisaccharide (Table II).

iv) Formation of formaldehyde. Formaldehyde was determined as was by Masamune and Yosizawa.[15] To 2 cc. of the reaction mixture in i) were added 1 cc. of 1 *N* HCl, 0.7 cc. of 1.2 *N* sodium arsenite, 0.7 cc. of 1 *M* sodium acetate and 0.5 cc. of a 8% (w/v) dimedone solution in 95% ethanol in sequence, and the mixture was heated in a boiling water-bath for 10 minutes and then stood in air for 1 hour. The crystalline deposit was filtered off on a glass-filter, washed, and weighed after drying at 85–95°C for 20 minutes. The formaldehyde dimedone derivative melted at 189–190°C. The trisaccharide gave 0.98 mole of formaldehyde per mole (Table II).

v) Determination of galactose. 1 cc. of the reaction mixture in i) was diluted with 10 cc. of water, shaken with a mixture of 2 g. each of Dowex 50 (H-form) and Dowex 1 (carbonate-form) for 15 minutes, and centrifuged. The supernatant containing floating resin particles was filtered, and the filtrate was subjected to galactose analysis after Masamune and Sakamoto.[5] In Table II are shown the amounts of remaining galactose, expressed in percentages of the trisaccharide, after various durations of oxidation. The galactose residue in the trisaccharide was attacked very slowly, but broken up almost in 72 hours.

Summary

1. A trisaccharide "gastro-N-trisaccharide" was separated in crystalline form from the group mucopolysaccharide which had been prepared by alkaline scission at room temperature of pig stomach mucus.

2. It was concluded that the trisaccharide has the structure of acetylglucosamine-4-galactosido-4-acetylgalactosaminide, being based on the results of oxidation experiments with hypoiodite and sodium metaperiodate and that of Morgan-Elson test.

The expenses of this work were defrayed by a grant given from the Ministry of Education through the Grant Committee for Scientific Researches. H. Masamune.

References

1) Kuhn, Gauhe & Baer, Chem. Ber., 1954, **87**, 1138.
2) Courtois, Fleury & Bieder, Bull. soc. chim. biol., 1953, **35**, 565.
3) Yosizawa, Tohoku J. Exp. Med., 1951, **54**, 115.
4) Masamune & Yosizawa, *ibid.*, 1956, **64**, 53.
5) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
6) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.
7) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.

8) Masamune, Hakomori, Numabe, Akama & Kamiyama, Tohoku J. Exp. Med., 1955, **61**, 283.
9) Kuhn & Kirschenlohr, Chem. Ber., 1954, **87**, 384.
10) Masamune & Yosizawa, Tohoku J. Exp. Med., 1953, **59**, 1.
11) Suzuki, J. Bioch. (Japan), 1938, **27**, 367.
12) Macleod & Robison, Bioch. J., 1929, **23**, 517.
13) Masamune & Yosizawa, Tohoku J. Exp. Med., 1954, **60**, 135.
14) Carlquist, Acta chem. Scand., 1948, **2**, 770.
15) Masamune & Yosizawa, Tohoku J. Exp. Med., 1951, **55**, 43.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Biochemical Studies on Carbohydrates

CLXXXVII. Oligosaccharides Separated after Acetolysis of the Group Mucopolysaccharide from Pig Gastric Mucus

Second Report: Acetylglucosamine-4-β-galactoside (Acetyllactosamine)

By

Hajime Masamune and **Zensaku Yosizawa**

(正宗 一) (吉沢 善作)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, October 30, 1955)

In the foregoing work[1], the group mucopolysaccharide from pig stomach mucus was acetolyzed, followed by deacetylation and fractionation by means of a cellulose powder column, and gastro-N-trisaccharide was isolated from Frs. 49–90 of the 90 fractions. The present account deals with acetylglucosamine-4-β-galactoside which has been separated in crystalline form from the 41st-48th fractions. It has $[\alpha]_D^{18}$ in water of 28.8° at equilibrium and melts at 169–171°C. It proved to be composed of equimolecular acetylglucosamine and galactose and gave phenyllactosazone. Yosizawa,[2] one of the writers, separated formerly this disaccharide from a similar material, though amorphous and not perfectly pure, and established its structure. Later on Tomarelli *et al.*[3] and Zilliken *et al.*[4] isolated it from commercial hog gastric mucin, and Kuhn and Kirschenlohr[5][6] from the blood group substance of meconium. The present substance showed the infrared spectrum identical with that of the synthetical product of Kuhn and Kirschenlohr.[6]

Experimental

Isolation of acetylglucosamine-4-β-galactoside

The 41st–48th of the 90 fractions of the deacetylated acetolysis products (starting material 20 g.) showed spots of galactose and two sugars of lower Rf values, when chromatographed with butanol-pyridine-water (5:3:2 by volume) according to Masamune and Yosizawa.[7] Those fractions were distilled together to syrupy consistency and redistilled with 30 cc. portions of abs. methanol, and the final dried residue was boiled with three 50 cc. portions of abs. methanol in succession (Each of the solutions was separated by centrifugation while warm). The solutions were united and distilled to 2–3 cc. (A few drops of octylalcohol were

used to evade foam formation), when crystals began to appear. The condensate was placed in an ice-chamber overnight. The deposit was collected on a suction funnel and washed with 3 cc. of ice-chilled abs. methanol, 5 cc. of abs. ethanol and 5 cc. of dry ether in turn. 0.4 g. was the yield here. The crude substance was recrystallized from abs. methanol three times in a similar manner. The product had the crystalline form in Fig. 1, and melted at 169–171°C on heating. Molisch positive, Morgan-Elson negative.

Properties of the disaccharide

1) When chromatographed with solvent butanol-pyridine-water (5:3:2 by volume) according to Masamune and Yosizawa[7] (Sliver Toyo Roshi filter paper no. 3), it moved with an average Rr (rate of flow as compared with rhamnose) value of 0.46 and an average Rg (rate of flow as compared with glucose) value of 0.76. The spot was colored orange-brown by the aniline hydrogen phthalate reagent of Partridge.

Fig. 1. Microphotograph of crystals of the disaccharide.

2) *Optical rotation.* 10.4 mg. of the substance were dissolved in 1 cc. of water at 18°C and examined as for its rotation in a 1 dm. tube.

Time after dissolution (min.)	α (°)	$[\alpha]_D^{18}=\frac{\alpha\times 100}{1\times 1.04}$ (°)
5	+0.50	+48.1
10	+0.48	+46.2
20	+0.44	+42.3
30	+0.42	+40.4
60	+0.37	+35.6
2×60	+0.32	+30.8
3×60	+0.30	+28.8
24×60	+0.30	+28.8

By extrapolation to time zero is obtained $[\alpha]_D^{18}$ of +51.5° as the initial rotation. The half value time of mutarotation is 32 minutes.

3) *Paper partition chromatography of the sugar components.* 10 mg. of the substance were hydrolyzed with 2 cc. of 0.5 *N* HCl in a sealed tube by heating at 100°C for 10 hours. The hydrolysate was transferred to a small dish and evaporated at 30–40°C in vacuo over solid $CaCl_2$ and NaOH. The residue was subjected repeatedly to dissolution in water and evaporation in order to expel off HCl as far as possible. The final dried residue was taken up in 0.2 cc. of water, and a 0.02 cc. portion of the solution was sampled on a paper sliver to chromatograph as described above. Glucosamine and galactose were identified (Fig. 2).

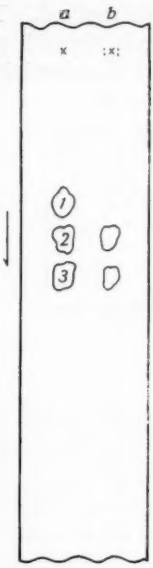


Fig. 2. Butanol-pyridine-water (5 : 3 : 2 by volume) -chromatogram of the hydrolysate of the disaccharide. Sliver 12×55 cm. Irrigation descending. Temperature and duration of test 24–26°C and 16 hrs. respectively. Indicator aniline hydrogen phthalate. The standard solution was 1% with respect to individual sugars hexosamine hydrochlorides and galactose.
a : reference run. b : main run. 1 : galactosamine. 2 : glucosamine. 3 : galactose.

4) *Quantitative analysis*. See Table I. The molecular weight calculated from the iodine use amounts to 423.

TABLE I

Composition of the Disaccharide

Figures express percentages.

Analysis	Found	Calcd. for $C_{14}H_{25}O_{11}N \cdot CH_3OH$ (415.4)
N*	3.33	3.37
C†	43.27	43.37
H†	7.01	7.03
Glucosamine‡	42.8	43.1
Galactose§	42.0	43.3
Acetyl//	10.1	10.4
Iodine use as glucose¶	42.6	43.3

* Micro Kjeldahl. † Pregl ; Kindly analysed by Mr. M. Suzuki in the Chemical Department. ‡ Masamune and Yosizawa.[8] § Masamune and Sakamoto.[9] // Suzuki.[10] ¶ Macleod and Robison.[11]

5) *Infrared spectrum* (The writers are indebted to Mr. Y. Ikegami in the Chemical Institute of Non-aqueous Solutions). The infrared absorptions were measured in Perkin-Elmer Model 21 recording infrared

spectrophotometer with a NaCl prism by the KBr disk method. The spectrum obtained is shown in Fig. 3, which coincides perfectly with that of 2-desoxy-2-acetamino-lactose synthesized by Kuhn and Kirschenlohr.[6]

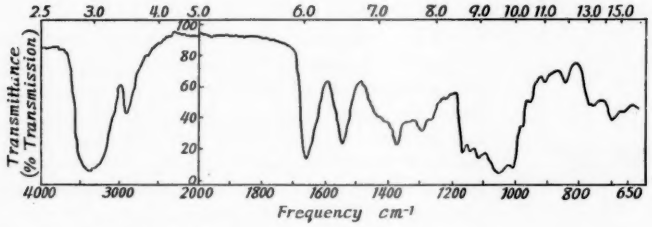


Fig. 3. Infrared spectrum of the disaccharide.

6) *Phenylosazone*. The procedure of Kuhn & Kirschenlohr[5] was applied. 100 mg. of the substance, 1 g. of phenylhydrazine hydrochloride and 0.5 g. of crystalline sodium acetate were dissolved together in 7 cc. of water, and heated for 1.5 hours in a boiling water-bath. The oily osazone was kneaded with water, and the centrifugate was taken up in 2–3 cc. of hot water and stood in air. 9 mg. of crystals appeared as yellow red needles. They were recrystallized three times. F.P. 203–205°C. N. (Friedrich's micro Kjeldahl method) 10.48% (Calcd. for $C_{24}H_{32}N_4$: 10.76%). Mixtures of the product with phenyllactosazone prepared with lactose showed no depression of melting point.

The expenses of this work were defrayed by a grant from the Education Department through the Grant Committee for Scientific Researches. H. Masamune.

References

1) Masamune, Yosizawa & Haga, Tohoku J. Exp. Med., this volume, p. 257.
2) Yosizawa, *ibid.*, 1949, **51**, 51; 1950, **52**, 111, 145.
3) Tomarelli, Hassinen, Eckhardt, Clark & Bernhart, Arch. Bioch. & Biophys., 1954, **48**, 225.
4) Zilliken, Smith, Tomarelli & György, *ibid.*, 1955, **54**, 398; Zilliken, Smith, Rose & György, J. Biol. Chem., 1954, **208**, 299.
5) Kuhn & Kirschenlohr, Chem. Ber., 1954, **87**, 560.
6) Kuhn & Kirschenlohr, *ibid.*, 1954, **87**, 1547.
7) Masamune & Yosizawa, Tohoku J. Exp. Med., 1953, **59**, 1.
8) Masamune & Yosizawa, *ibid.*, this volume, p. 53.
9) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
10) Suzuki, J. Bioch. (Japan), 1938, **27**, 367.
11) Macleod & Robison, Bioch. J., 1929, **23**, 517.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Biochemical Studies on Carbohydrates

CLXXXVIII. Chemical Nature of KIK Factors

Second Report: KIK Factor in Cancerous Ascitic Fluid*

By

Koretaka Sugimoto
(杉本 是孝)

From the Medico-chemical Institute, Tohoku University, Sendai;
Director: Prof. H. Masamune

(Received for publication, October 30, 1955)

The KIK factor was separated from cancerous ascitic fluids of patients with the primary seat in stomach or in liver. The preparations were glucidamins and resembled one another in composition and other properties, indicating their identity. Each caused 10% decrease of blood erythrocytes number when 0.1 mg. of it was injected per kg. body weight intraperitoneally into a rabbit.

Corresponding substances were obtained from ascitic fluids that accumulated due to liver cirrhosis and those due to tuberculosis. The substance from the former source differed in rotation and electrophoretic mobility and that from the latter source not only in rotation and electrophoretic mobility but also in tests and analyses from the KIK factor above, and they were both devoid of the anemia-inducing potency.

To add, the KIK factor from cancerous ascitic fluids (KIK factor III) is identical with neither of KIK factors I and II in cancerous gastric juice,[2] namely, a common partial structure among the various KIK factors was suggested.

Experimental

Isolation of the KIK Factor from Cancerous Ascitic Fluids and of Corresponding Substances from the Non-cancerous

1) KIK factor from combined cancerous ascitic fluids of a patient with primary seat in stomach. 3.2 *l.* of the fluids (yellow and translucent, sp. gr. 1.020, pH 7.8, Rivalta positive) were filtered to remove floating exfoliated tissue particles, and solid trichloroacetic acid was added to the filtrate to 2.5%. After standing overnight, the precipitate was centrifuged off and washed with two 100 cc. portions of 2.5% trichloroacetic acid.

* A half of the article read before the 14th General Meeting of the Japanese Cancer Association, Kyoto, April 2-4, 1955[1] is corrected in part.

The supernatant (800 cc.) and washing were dialyzed together against running water for 5 days and distilled in vacuo to a few cubic centimeters and excessive alcohol was added. The flocculent deposit was separated and washed with alcohol and ether in turn. 180 mg. of a white powder were given. It was taken up in 18 cc. of water, freed from the insoluble part and electrodialyzed in a Pauli apparatus against changes of distilled water, reversing the current direction from time to time. The electrodialysis was continued for 5 days until no more precipitate occurred. The centrifuged non-colored supernatant was condensed to about 1 cc., precipitated with alcohol and dried after washing with alcohol and ether. A white powder weighing 54 mg. (17 mg. per liter of the ascitic fluids started with) was yielded.

Batches of ascitic fluids from six other stomach cancer patients were subjected to the procedure in 2) below. Their volumes were 3, 1, 4, 3.8, 4 and 4 *l.*, and 50, 20, 60, 56, 80 and 80 mg. of the KIK factor were given. The first four products and the one above were combined together (**III$_1$**), and the last two were separately preserved (**III$_1$'** and **III$_1$''**).

2) KIK factor from united cancerous ascitic fluids of a patient with original seat in liver. 4.2 *l.* of the fluids (yellowish brown and translucent, sp. gr. 1.018, pH 8.4, Rivalta positive) were filtered, and the filtrate was distilled in vacuo to about 1/10 the volume and after 5 days' dialysis, electrodialyzed similar to above. The centrifuged supernatant was distilled under reduced pressure to dryness. And the still-residue (greenish colored, 10 g.) was pulverized and homogenized in 1 *l.* of water. The green solution given was freed from the insoluble part, and the substance precipitable by trichloroacetic acid at 2.5% was separated and washed as above. The mother fluid and washing were dialyzed for 2 days, condensed to about 2 cc. and precipitated with alcohol, followed by washing with alcohol and ether. The white powder given weighed 70 mg. (17 mg. per liter of the ascitic fluids).

The same procedure was applied to 1 and 1.4 *l.* batches of ascitic fluids from two other hepatoma patients, and 20 and 30 mg. of products were yielded, which were put together with the one above—**III$_2$**.

3) The fraction corresponding to the KIK factor above of united ascitic fluids of a liver cirrhosis patient. 1.4 *l.* of the fluids (yellowish-brown and transparent, sp. gr. 1.011, pH 8.2, Rivalta negative) were subjected to the procedure in 2), and 30 mg. (21 mg. per liter of the fluids) of a white powder were given as the product.

Fractioned in a similar manner, 5 *l.* of combined ascitic fluids from another liver cirrhosis patient gave 110 mg. of a product which was united with the one above—**Fr$_{Ic}$**.

4) The fraction corresponding to the KIK factor above of combined

ascitic fluids of a tuberculosis patient. From 3 *l.* of the fluids (yellow and turbid, sp. gr. 1.028, pH 8.8, Rivalta positive) were yielded 80 mg. (27 mg. per liter of the ascitic fluids) of a white powder by the procedure in 2).

From 6 *l.* in total of the ascitic fluids of another tuberculosis patient were obtained 120 mg. of a product in the same manner. It was united with the one above—**Fr_{tb}**.

Properties of the Preparations

Biological property

Assay for anemia-inducing potency. It was processed as described in the First Report[2], employing rabbits weighing 2.0–3.5 kg. Preps. III_1 and III_2 caused 10% decrease in number of the erythrocytes when injected in the dose of 0.1 mg. per kg. body weight into a rabbit, while Preps. Fr_{lc} and Fr_{tb} were inefficient. (Table I) It is noteworthy that the potency of Prep. III_1 decreased remarkably, when the substance was boiled with abs. alcohol or benzene in advance, but not when the substance was boiled with water (Table I).

Physical properties

1) *Electrophoretic examination.* Acidic and alkaline solutions of the preparations were examined in a Tiselius apparatus. All of them gave a single boundary. KIK factor preparations III_1 and III_2 showed similar mobilities, but Fr_{lc} and Fr_{tb} differed from them as well as from each other in this respect. (Fig. 1)

2) *Optical rotation in water.*

$$III_1: \quad [\alpha]_D^{15} = \frac{-0.215 \times 1}{0.005 \times 1} = -42.9^\circ \text{ (pH of the solution 5.8)}$$

$$III_2: \quad [\alpha]_D^{15} = \frac{-0.103 \times 1}{0.0025 \times 1} = -41.3^\circ \text{ (pH of the solution 5.6)}$$

$$Fr_{lc}: \quad [\alpha]_D^{15} = \frac{-0.129 \times 1}{0.0025 \times 1} = -51.6^\circ \text{ (pH of the solution 5.8)}$$

$$Fr_{tb}: \quad [\alpha]_D^{15} = \frac{-1.054 \times 1}{0.0111 \times 1} = -94.9^\circ \text{ (pH of the solution 7.0)}$$

III_1 and III_2 are resembling in rotatory power, but Fr_{lc} and particularly Fr_{tb} diverge from them.

3) *Solubility.* The preparations showed the same solubilities and precipitabilities. They are all readily soluble in water of any pH, but insoluble in abs. methanol, abs. ethanol, ether, acetone, 90% phenol, benzene, the sat. $(NH_4)_2SO_4$, the sat. Na_2SO_4 and glacial acetic acid. They are not precipitated from water by cupric chloride, barium acetate, baryta and lead acetate.

Chemical properties

TABLE

Anemia-inducing Potency

Rabbits Nos. 6, 7, 8 and 9 were used at intervals of more than 3 months.

Assay No.	Rabbits			Material injected	Injection dose per kg. body wt.
	No.	Body wt. (kg.)	Sex		
1	1	2.6	♂	III_1	2
2	2	3.5	"		"
3	3	2.1	"		"
4	4	3.1	"		1
5	5	2.0	"		"
6	6	2.4	"		"
7	7	3.2	"		0.1
8	8	2.4	♀		"
9	9	3.1	"		"
10	7	3.5	♂	III_2	1
11	10	2.5	"		0.1
12	11	3.1	"		"
13	12	3.1	♀		"
14	13	2.2	♂	Fr_{Ic}	5
15	14	2.5	"		3
16	9	3.3	♀		1
17	8	2.6	♀	Fr_{tb}	5
18	15	2.6	♂		"
19	16	3.4	♀		2
20	17	3.0	♀	III_1 boiled with alcohol*	1
21	18	2.4	♂		"
22	19	2.8	"		"
23	20	3.0	♀	III_1 boiled with benzene†	2
24	21	3.0	♂		1
25	8	2.4	♀	III_1 boiled with water‡	1
26	22	2.5	♂		0.1
27	6	2.5	"		"

* 15 mg. of III_1 were suspended in 10 cc. of abs. alcohol and boiled under reflux for 3 benzene and refluxed for 3 hours, followed by recovery as in *. ‡ 10 mg. of III_1 were

1) *Test-tube tests.* The results are embodied as follows:

Reaction	Preparation					
	III_1	III_1'	III_1''	III_2	Fr_{Ic}	Fr_{tb}
Molisch	##	##	##	##	##	++
Biuret	+*	+*	+*	+*	+*	++†
Osaki-Turumi						
Indirect	+	+	+	+	+	−
Direct	+	+	+	+	+	−
Bial-Mejbaum‡	+	+	+	+	+	±

* Red violet coloration. † Violet coloration, ‡ Red violet coloration.

I
of the Preparations

Number of red cells								
Before inj.	Time (hrs.) after inj.						Maximum fluctuation (%)	
	1	2	3	4	5	6	Individual	Average
592	596	574	491	469	**437**	408	−26.2	
567	556	552	470	453	**440**	480	−22.4	−25.8
508	464	430	403	**361**	368	459	−28.9	
598	558	477	462	428	**421**		−29.6	
436	397	389	364	**330**	340		−24.3	−22.1
400	384	390	380	**351**	366		−12.3	
633	582	544	580	**531**	544		−16.1	
573	532	559	553	553	**531**		− 7.3	−11.8
686	629	645	623	**604**	612		−11.9	
671	651	664	624	581	**574**	580	−14.3	
586	577	560	543	544	**530**	541	− 9.5	
549	564	524	498	**472**	508		−14.0	−12.0
550	567	528	505	**482**	518		−12.4	
566	566	572	561	**543**	551		− 4.1	
550	548	562	573	**580**	570		+ 5.4	
518	513	519	534	**539**	518		+ 4.1	
513	514	511	514	510	515		0	
644	615	**610**	645	625	630		− 5.3	
812	796	822	**786**	826	848		− 3.2	
527	538	549	**551**	541	530		+ 4.5	
617	617	616	622	**603**	616		+ 2.3	
655	635	615	610	**603**	612		− 7.9	
605	613	600	**593**	605	599		− 1.9	
655	653	648	641	**620**	649		− 5.3	
519	510	502	479	**455**	499		−12.3	
531	520	477	480	**475**	514		−10.5	−10.9
546	534	522	503	**484**	530		−11.3	

hours, followed by distillation in vacuo. † 10 mg. of III_1 were suspended in 3 cc. of dissolved in 3 cc. of water, refluxed for 1 hour and recovered as in *.

2) *Paper partition chromatography.* a) sugar components. 15 mg. of substance were hydrolyzed with 1 *N* H_2SO_4 by heating in an ampoule at 100°C for 5 hours. The hydrolysate was neutralized with the sat. baryta to pH 5.8 and centrifuged, and the centrifugate was washed with hot water three times. The supernatant and washings were distilled together in vacuo to a small volume, transferred to a dish and dried up in vacuo over solid caustic soda. The evaporated residue was taken up in 0.1 cc. of water, and two 0.03 cc. portions were sampled separately on different slivers of Toyo Roshi filter paper no. 2. Chromatograms were developed

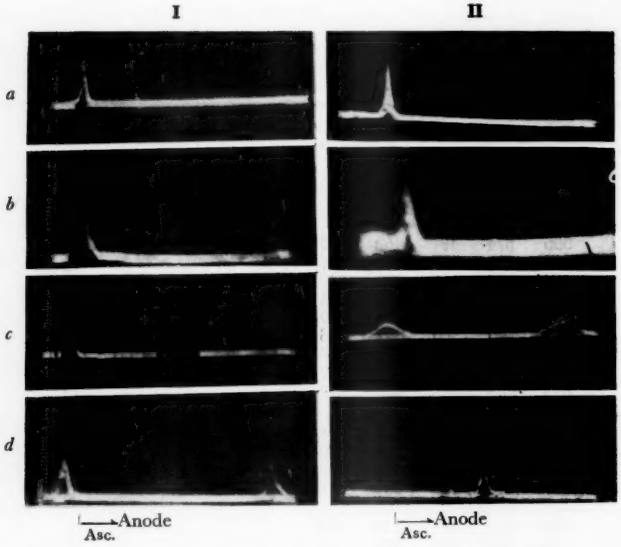


Fig. 1. Electrophoretic patterns (4/5×) of solutions of III_1 (*a*), III_2 (*b*), Fr_{1c} (*c*) and Fr_{tb} (*d*) in acetate buffer pH 4.6, I 0.2 (**I**) and phosphate buffer pH 7.8, I 0.2 (**II**); current 8 mA; temperature 20°C. Exposure 30 or 60 minutes after starting current (See below). Regarding the concentration of the solutions, see also below. Mobility:

III_1 (1%, pH 4.6): $\frac{-0.3\times0.0070\times0.3}{3600\times0.008}=-2.19\times10^{-5}$ cm²/volt/sec

(1%, pH 7.8): $\frac{-0.5\times0.0091\times0.3}{3600\times0.008}=-4.81\times10^{-5}$ "

III_2 (1%, pH 4.6): $\frac{-0.3\times0.0070\times0.3}{3600\times0.008}=-2.19\times10^{-5}$ "

(1%, pH 7.8): $\frac{-0.5\times0.0091\times0.3}{3600\times0.008}=-4.81\times10^{-5}$ "

Fr_{1c} (1%, pH 4.6): $\frac{-0.4\times0.0070\times0.3}{3600\times0.008}=-2.92\times10^{-5}$ "

(0.5%, pH 7.8): $\frac{-0.45\times0.0091\times0.3}{1800\times0.008}=-8.53\times10^{-5}$ "

Fr_{tb} (1%, pH 4.6): $\frac{-0.1\times0.0073\times0.3}{3600\times0.008}=-0.83\times10^{-5}$ "

(1%, pH 7.8): $\frac{-1.6\times0.0091\times0.3}{3600\times0.008}=-1.52\times10^{-4}$ "

in the descending way one with solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1 by volume) and the other with solvent butanol-pyridine-water (5:3:2 by volume) according to Masamune and Yosizawa.[3] Indicator the aniline hydrogen phthalate reagent of Partridge. Reference

runs were carried out with standard solutions which were 1% with respect to individual sugars. As shown in Fig. 2, galactosamine, glucosamine, galactose, glucose, mannose, xylose and/or lyxose and L-fucose were detected in any of the hydrolysates of the preparations.

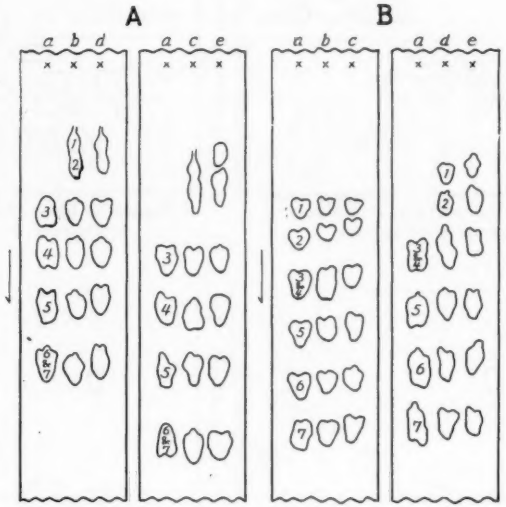


Fig. 2. Sugar paper chromatograms of III_1, III_2, Fr_{Ic} and Fr_{Ib}. Paper slivers: Toyo Roshi filter paper no. 2, 60×12 cm. Irrigation descending. Temperature and duration of test 24°±1°C and 20 hrs. respectively. (**A**) developed with solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1 by volume). (**B**) developed with butanol-pyridine-water (5:3:2 by volume).
a: reference run, b: III_1, c: III_2, d: Fr_{Ic}, e: Fr_{Ib}. 1: galactosamine, 2: glucosamine, 3: galactose, 4: glucose, 5: mannose, 6: xylose, 7: L-fucose. (Lyxose and xylose overlap each other, whether irrigation is effected with the first solvent or with the second.)

b) Amino acid components. 8 mg. of substance were heated with 0.8 cc. of 6 *N* HCl in a sealed tube at 100°C for 24 hours. The humin produced was centrifuged off, and the supernatant was evaporated in vacuo over solid caustic soda. The residue was subjected to dissolution in water and evaporation repeatedly to expel off most of the hydrochloric acid. The final residue was taken up in 0.1 cc. of water, and 0.04 cc. of the solution was chromatographed by the procedure of Masamune and Hakomori,[4] replacing a solvent phenol-*M*/20 borax (8:2 by volume) or phenol-*M*/10 borax (50:7 by volume) for solvent phenol-*M*/10 borax (86:14 by volume) in the secondary irrigation. Indicator 0.1% nin-

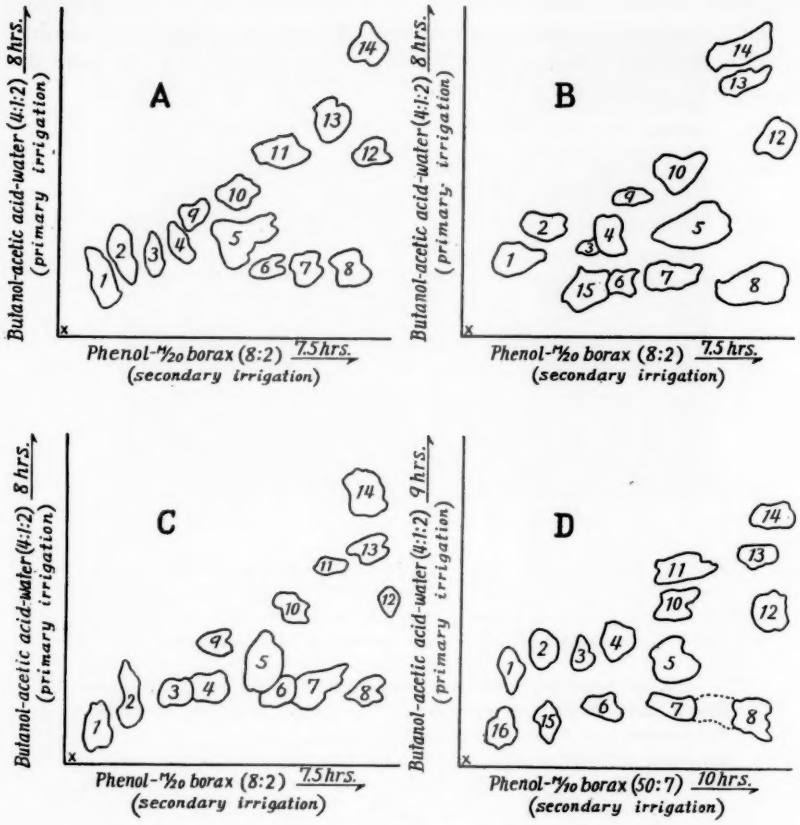


Fig. 3. Amino acid paper chromatograms of III_1 (**A**), III_2 (**B**), Fr_{1c} (**C**) and Fr_{tb} (**D**). Paper squares used: Toyo Roshi filter paper no. 2, 30× 30 cm. Temperature: (**A**)-(**C**) 22°C; (**D**) 25°C.

1: aspartic acid, 2: glutamic acid, 3: serine, 4: glycine, 5: hexosamine, 6: lysine, 7: histidine, 8: arginine, 9: threonine, 10: alanine, 11: tyrosine, 12: proline, 13: valine and/or methionine, 14: lecuine and/or isoleucine, 15: cystine, 16: a peptide.

hydrin in butanol saturated with water. (Fig. 3 (A, B, C, D)) Any of the preparations examined was found to contain aspartic and glutamic acids, serine, glycine, histidine, arginine, lysine, proline, alanine, leucine and/or isoleucine and valine and/or methionine, and III_1 and Fr_{1c} threonine and tyrosine, III_2 threonine and cystine and Fr_{tb} tyrosine and cystine in addition. Since threonine, tyrosine and cystine are liable to decom-

position by hot strong acids particularly in the presence of sugars, the discrepancies among the preparations may be a mere show.

3) *Quantitative analysis.* See Table II.

TABLE II

Composition of the Preparations

Analysis	In per cent						In equivalents per equivalent weight					
	III_1	III_1'	III_1''	III_2	Fr_{1c}	Fr_{1b}	III_1	III_1'	III_1''	III_2	Fr_{1c}	Fr_{1b}
N*	8.0	7.1	7.7	7.0	8.0	10.7	9.3	8.2	9.2	7.8	9.3	14.1
Hexosamines as glucosamine†	11.0	11.1	10.6	11.5	11.0	9.5	1.0	1.0	1.0	1.0	1.0	1.0
Galactose‡	6.9	7.5	6.0	7.6	7.0	5.7	0.6	0.7	0.6	0.7	0.6	0.7
Glucose‡	4.0	4.6	4.3	5.9	3.2	3.5	0.4	0.4	0.4	0.5	0.3	0.4
Mannose‡	6.9	7.0	7.3	7.8	5.5	5.0	0.6	0.6	0.7	0.7	0.5	0.5
L-Fucose§	0.5	0.6	0.7	0.9	0.7	0.4	0.05	0.06	0.07	0.08	0.07	0.05
Xylose and/or lyxose as xylose//	1.8	1.9	1.6	1.8	2.1	1.2	0.19	0.20	0.19	0.19	0.23	0.15
Sialic acid¶	3.6	4.6	3.6	3.4	3.2	1.0	0.19	0.24	0.20	0.17	0.17	0.06
Ash**	1.5	1.8	1.2	2.5	2.4	2.2						

* Micro Kjeldahl. † Masamune and Yosizawa.[5] ‡ Masamune and Sakamoto.[6] § Masamune and Sakamoto.[7] // Masamune and Sakamoto.[8] ¶ Werner and Odin (the direct Ehrlich reaction)[9]; Calcd. as $C_{11}H_{19}NO_9$.[10] ** Pregl method without use of H_2SO_4.

SUMMARY

1. A KIK factor (III) was isolated from cancerous ascitic fluids and corresponding substances from the non-cancerous in electrophoretically pure or almost pure state.

2. They were investigated regarding anemia-inducing potency and physical and chemical properties.

3. They were found to be glucidamins, and contained galactosamine, glucosamine, galactose, glucose, mannose, L-fucose, xylose and/or lyxose, sialic acid and many amino acids.

4. The KIK factor in cancerous ascitic fluids differs in rotation and electrophoretic mobility or in rotation, electrophoretic mobility and composition from the corresponding substances in non-cancerous fluids which are devoid of the anemia-inducing potency.

5. The KIK factor from cancerous ascitic fluids differs from either of the two KIK factors in cancerous gastric juices.

Thanks are due to the Ministry of Education which gave a grant to us through the Grant Committee for Scientific Researches. H. Masamune.

References

1) Masamune, Sugimoto & Kawasaki, Gann, 1955, **46**, 126.
2) Masamune & Kawasaki, Tohoku J. Exp. Med., 1956, **63**, 369.
3) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
4) Masamune & Hakomori, *ibid.*, 1956, **64**, 59.
5) Masamune & Yosizawa, *ibid.*, 1956, **64**, 53.
6) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
7) *Idem*, *ibid.*, 1956, **63**, 363.
8) *Idem*, *ibid.*, 1956, **63**, 357.
9) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.
10) Cf. Werner & Blix, 3 ème congrès international de biochimie, Bruxelles, août 1–6, 1955, Résumés des communications, p. 3.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Biochemical Studies on Carbohydrates

CLXXXIX. N-Glycoside- and Acetal-Linkage in the Molecule of the Group Mucopolysaccharide from Pig Stomach Mucus*

By

Hajime Masamune, Sen-itiroh Hakomori
(正宗 一) (箱守 仙一郎)
and **Osamu Masamune**
(正宗 研)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, October 30, 1955)

Regarding the union between the carbohydrate and polypeptide moieties in Molisch-positive mucopolysaccharides and glucidamins,[1] Masamune,[2] one of the writers, ascribed it formerly to normal co-valencies because of the incapability of separating the moieties from each other without breaking up into their components. In 1942, Stacey and Wooley[3] assumed the ester linkage between a carboxyl of the amino acid-grouping and a hydroxyl in the carbohydrate moiety of ovomucoid, on the basis that the latter part was easily separated by treatment with alkali, and recently Werner[4] imagined an O-glycosidic link connecting the reducing end of the carbohydrate chain and the hydroxyl of threonine in L-fucose-containing mucopolysaccharides ("fucomucan") from tracheal, gastric, intestinal and cervical mucus.

In 1952, Masamune[5] suggested N-glycosidic linkage between the carbohydrate and polypeptide moieties in the molecule of group lipids and group mucopolysaccharides, because the number of free amino and carbonyl groups increased, when those substances were treated with a mild acid. As to the group lipids, Hakomori[6] furnished later several facts in support of Masamune's view, but the case of the group mucopolysaccharides awaits a comprehensive study, since weak acid-labile peptide- and O-glycoside-linkages have been demonstrated in mucopolysaccharides by Kabat[7] and other investigators[8]-[10]. We have examined the group mucopolysaccharide from pig stomach mucus, and established the presence of N-glycosidic, esteric and ethereal linkages between its two moieties, of which the N-glycosidic will be accounted for here together with an acetal link within the carbohydrate moiety.

* A detailed note of what was read by Masamune before the 3rd International Congress of Biochemistry, Brussels, August 1-6, 1955, with partial correction.

The experiments and comments will be described item by item (Cf. Tables IIA and IIB).

1) The group mucopolysaccharide obtained after alkaline scission of pig stomach mucus (MP I) was oxidized with sodium hypoiodite. The product recovered after dialysis (MP I′) contained one molecule of hexosamine less than MP I for a unit structure corresponding to the minimum molecular weight (unit molecule), but it still gave the positive indirect-Osaki-Turumi reaction with development of the red color of an intensity reaching half that of the color given by the mother substance which corresponded to two N-acetylglucosamine molecules. Hence it is plausibly assumed that MP I has two terminal N-acetylhexosamine residues owing to branching of the carbohydrate chain, of which one is bound N-glycosidically at C_I as surmised from the fact that glycylglycine ethylester-N-acetylglucosaminide[6] and other resembling N-acetylglucosaminides are indirect-Osaki-Turumi positive despite of their stableness against hypoiodite.

2) Portions of MP I were heated with 1 *N* acetic acid at 100°C for various durations of time, followed by dialysis. The undialysable product after 1.5 hours' heating (MP Ia) contained one molecule each of glucosamine, L-fucose, sialic acid and aspartic acid less than MP I. The components cleft off were found in the dialysate (Acetylglucosamine was detected instead of glucosamine). The undialyzable product after 3 hours' heating contained lesser amounts of not only hexosamine and L-fucose but also galactose and several amino acids than the corresponding product above, and in the dialysate here were found galactose, glycine, alanine and leucine and/or isoleucine besides those components in the dialysate above. MP Ia used as much iodine as corresponded to approximately one hexose unit.

3) MP Ia was oxidized with alkaline iodine and dialyzed. The product recovered from the fluid in the dialysis bag (MP Ia′) was one hexosamine molecule less than MP Ia, the other components being retained as such.

4) MP Ia and I were converted into DNP-derivatives according to Sanger[11] and hydrolyzed. The hydrolysate of the DNP-MP Ia contained ε-DNP-lysine and DNP-glutamic acid, whereas the hydrolysate of DNP-MP I had ε-DNP-lysine as a sole dinitrophenylated amino acid. As already mentioned (2) above), MP I lost the aspartic acid residue when changed into MP Ia. Therefore, it is taken for granted that aspartic acid is bound with amino group of glutamic acid by its carboxyl on one hand and with C_I of a terminal acetylhexosamine by its amino group on the other, and is split from both the glutamic acid and acetylhexosamine residues by acetic acid.

5) MP I was dissolved in a 2% mercuric chloride solution and kept at 60°C for 30 minutes, followed by dialysis. The analysis of the undialyzed product (MP Ib) revealed that it was one glucosamine molecule less than the mother substance and lacked sialic acid but no decrease occurred in other sugar components. Namely, those molecules of glucosamine (acetylglucosamine) and sialic acid are each fixed by acetal union, but L-fucose and other components easily separable with dilute acetic acid are bound by other kinds of link. MP Ib showed indirect-Osaki-Turumi reaction corresponding to 1 acetylglucosamine molecule per unit molecule. It was further noticed that MP Ia and Ib gave pretty less viscous solutions than MP I.

N-Glycoside- and acetal-link are generally unstable against acid. However, as described in 2) and 3), only one of the acetylglucosamine residues in MP I became dialyzable by 1.5 hours' treatment with acetic acid, and oxidation of the product thereby (MP Ia) with hypoiodite gave the product MP Ia′ which has only one hexosamine molecule less than MP Ia. It might be most probable that the two terminal acetylhexosamine molecules, one reducing and the other non-reducing, in MP I are bound to C_1 of a sugar residue, presumably a galactose residue, by acetal union, and the cleavage by the acid occurs stepwise in the way that the acetylglucosamine rest holding the free carbonyl is split off firstly and secondly the remaining of the bonds participating in the acetal union changes into the O-glycosidic, with following fission of the N-glycosidic link.

The formulas of MP I and its derivatives in Fig. 1 will elucidate well all the findings above. Moreover, judging from the analytical figures in Table IIB, more than half of the amino acids in the substance must vary from one unit molecule to another and such amino acids as aspartic and glutamic acids, serine and alanine only are always present in the units.

Experimental

Analytical methods

Hexosamine was estimated by a minor modification of Blix method[12] and the indirect-Osaki-Turumi positive acetylhexosamine residues after Yosizawa.[13] Galactose and L-fucose were determined according to Masamune and Sakamoto,[14),15] sialic acid by the direct-Ehrlich- and orcinol-reaction according to Werner and Odin,[16] amino acids according to Masamune *et al.*,[17] and iodine use according to Macleod and Robison.[18] The minimum molecular weight of MP I was computed from the iodine use but those of its derivatives from the number of galactose molecules which remains undecreased.

Material

The Material MP I was prepared from pig stomach mucus as described by Yosizawa.[19] Although a widely spread boundary was given on electrophoresis as shown in Fig. 2, it was proved to be practically homogeneous by

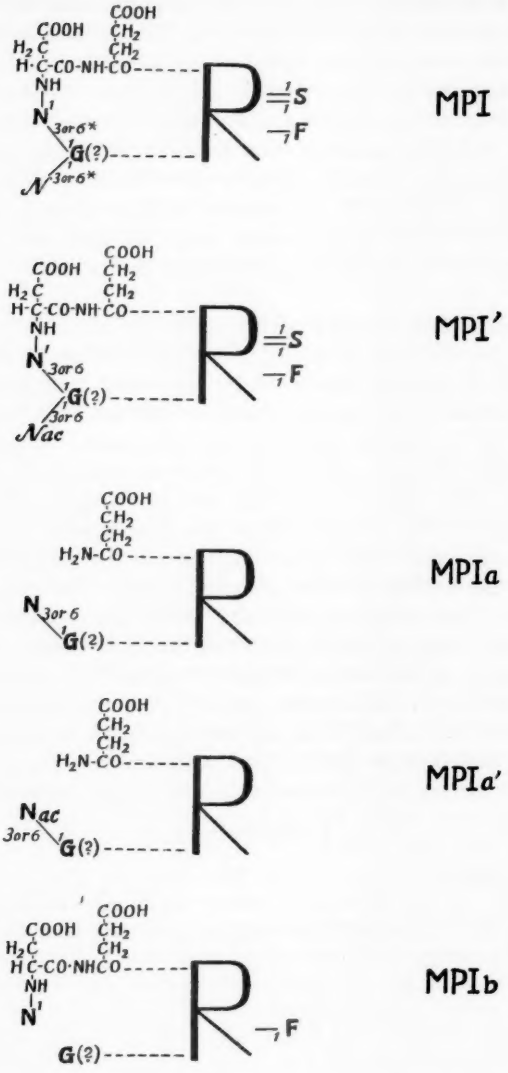


Fig. 1. Showing structures of the reducing terminal and neighbourhood in the unit molecule of MP I and its derivatives. **N**: N-acetylhexosamine. *N*: N-acetylglucosamine. **N**ac: N-acetylhexosaminic acid. *N*ac: N-acetylglucosaminic acid. **G**: galactose, **F**: L-fucose, **S**: sialic acid. * Cf. Kuhn and Kirschenlohr.[11a)]

chemical analysis of fractionated alcoholic precipitates and of divisions given by electrophoretic sedimentation method of Masamune *et al.*,[20] as illustrated in Table I.

TABLE I

Comparison of Fractionated Precipitates by Alcohol and of Divisions by Electrophoretic Sedimentation of MP I

The electrophoretic fractionation was carried out in acetate buffer of pH 4.6 and I 0.2; temp. 15–16°C; current 10 mA; duration of time 20 hrs. Regarding the analytical methods, see the text. Figures express percentages.

	Fraction	N	Galactose	Hexosamines as glucosamine	L-Fucose
Fractionation w. alcohol	Ppt. by alcohol at 0–75%	4.3	25.6	35.4	12.3
	Ppt. by alcohol at 75–90%	4.1	26.3	34.2	11.4
Electrophor. fractionation	Fraction pulled out of the tube	4.1	25.8	36.9	12.2
	Fraction remaining in the tube	4.0	26.6	35.0	13.1

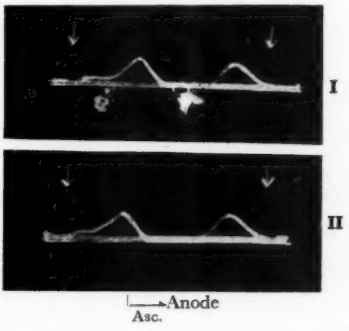


Fig. 2. Electrophoretic patterns (4/5×) of 1% solutions of MP I in phosphate buffer of pH 7.7 and I 0.2 (**I**) and acetate buffer of pH 4.6 and I 0.2 (**II**); current 8 mA; temperature 15°C. Exposure 60 minutes after starting current. ↓ starting boundary.

The analytical figures of the material are given in Table II (A, B), which indicate that the unit structure corresponding to the minimum molecular weight of 3500 is composed of seven molecules of hexosamine, five molecules of galactose, two molecules of L-fucose and one molecule each of sialic acid, aspartic acid, glutamic acid, serine and alanine. The other amino acid components glycine, threonine, histidine, arginine, lysine, proline, valine, leucine and/or isoleucine were still less. The amount of amino acids changed neither by prolonged electrodialysis nor by passing through a column of Dowex 50 (H-form) of the solution

of the material, so that they were essential constituents but not contaminations.

The substance had group potencies shown in Table III.

Preparation and composition of MP I'

200 mg. of MP I were dissolved in 60 cc. of water, added to with 60 cc. of $N/50$ iodine and 3 cc. of 5% sodium carbonate and stood at 25°C for 30 minutes. Then the reaction mixture was acidified with 0.5 N H_2SO_4, dialyzed in a cellophane bag against running water for two days until SO_4-ions and iodine disappeared, and after distilling in vacuo to a syrup, precipitated with abs. alcohol. The deposit was washed with ether and dried in vacuo over $CaCl_2$. 180 mg. of an amorphous white powder were given—MP I'.

Its electrophoretic mobility was nearly the same as that of MP I. As is shown in Table II, the carbohydrate moiety contained roughly six molecules of hexosamine, five molecules of galactose, two molecules of L-fucose and one molecule of sialic acid per unit molecule.

TABLE IIA

Composition of MP I and Its Derivatives

	Analysis		N	Hexosamines as glucosamine	Galactose	L-Fucose	Sialic acid	Aspartic acid	Glutamic acid	Indirect Osaki-Turumi reaction as acetylglucosamine	Iodine use as glucose	Min. mol. wt.	Ash
In per cent		MP I	4.3	36.1	26.6	11.5	7.3	2.9	4.2	9.8	5.1	3500*	0.8
	Derivative after treatment	of MP I w. alkaline iodine (MP I')	4.3	31.3	27.0	11.9	7.1			4.8	0.0	3450†	3.2
		of MP I w. hot 1 N CH_3COOH for 1.5 hrs. (MP Ia)	4.0	35.9	29.2	7.3	0.3	0.8	4.9		7.5	3200†	0.5
		of MP Ia w. alkaline iodine (MP Ia')	4.1	30.2	29.0	7.0					0.0	3200†	4.6
		of MP I w. $HgCl_2$ (MP Ib)	3.9	36.1	32.1	13.0	0.2			7.4	7.8	2900†	2.2
In number of molecules per unit molecule		MP I		6.9	5.2	2.4	0.8	0.8	1.0	1.6	1.0		
		MP I'		5.9	5.2	2.4	0.7			0.7	0.0		
		MP Ia		6.3	5.2	1.4	0.03	0.3	1.1		1.2		
		MP Ia'		5.4	5.2	1.3					0.0		
		MP Ib		5.9	5.2	2.2	0.02			1.0	1.2		

* Calculated from iodine use. † Calculated under the assumption that the substance contained 5.2 molecules of galactose per unit molecule.

Table IIB

Amino Acids Contents of MP I

4.53 mg. of the substance were dissolved in 0.5 cc. of 1 *N* HCl and heated together with 150 mg. of Dowex 50 (H-form) in a sealed tube at 100°C for 48 hours. The resin was filtered off under suction and washed with 1 *N* HCl and water, and the filtrate and washings were subjected to the analytical procedure of Masamune *et al.*,[17] after expelling off of most of the HCl in the manner usual in this Institute.

Analysis	In per cent	In number of molecules per unit molecule of MP I
Aspartic acid	2.9	0.78
Glutamic acid	4.2	1.00
Serine	2.5	0.83
Glycine	0.5	0.23
Threonine	1.2	0.35
Alanine	2.2	0.86
Valine	0.8	0.24
Proline	1.8	0.54
Leucine and/or isoleucine as leucine	0.3	0.08
Lysine	0.2	0.05
Histidine	0.1	0.02
Arginine	0.3	0.06

Table III

Anti-hemagglutinative Potencies of MP I

The assay was processed by Masamune and others' glass plate method.[21] — no aggl., ± faint aggl., + slight aggl., ++ marked to strong aggl., Goat : normal anti-O goat serum, Anti-OS : anti-OS saliva chicken serum. The antisera were diluted to titre 16 before use.

Serum	Red cells	Dilution of the substance (1:)											
		10^3	2×10^3	4×10^3	8×10^3	1.6×10^4	3.2×10^4	6.4×10^4	1.25×10^5	2.56×10^5	5.02×10^5	1.004×10^6	2.008×10^6
α	A	−	−	−	−	−	−	−	−	−	−	±	+
β	B	+	+	+	++	++	++	++	++	++	++	++	++
Goat	O	−	−	−	−	−	±	+	+	++	++	++	++
Anti-OS	O	−	−	−	−	−	−	±	+	++	++	++	++

Hydrolysis of MP I with 1 *N acetic acid*

Two 500 mg. portions of MP I were dissolved each in 25 cc. of 1 *N* acetic acid, and heated in a boiling water-bath one for 1.5 hours and the other for 3 hours. The hydrolysates were dialyzed against five changes of distilled water and distilled in vacuo almost to dryness, and excessive alcohol was added. The

deposits were washed with dry ether and dried in vacuo.

As is shown in Table II, the product after 1.5 hours' hydrolysis (MP Ia) contained approximately six molecules of hexosamine, five molecules of galactose, one molecule each of L-fucose and glutamic acid, and mere small amounts of sialic and aspartic acids, that is, one molecule each of hexosamine, L-fucose and sialic and aspartic acids were cleft off by the hydrolysis. It looks that the molecule of N-acetylhexosamine constituting the reducing terminal in MP I was split off and at the same time a new reducing terminal formed as a result of cleavage of the N-glycosidic link. Analytical figures of the product after 3 hours' hydrolysis indicated it to comprise lesser hexosamine, L-fucose, galactose, glutamic acid and other amino acids than MP Ia.

Examination of the dialysate of the acetic acid-hydrolysates of MP I

The dialysate of each of the hydrolysates was distilled in vacuo to about 50 cc. and passed through a 20×1.5 cm. column of Dowex 50 (H-form). The column was subsequently washed with water. The effluent and washing were concentrated together in vacuo to dryness and dissolved in 0.5 cc. of water. Two 0.01 cc. portions of the solution were paper-chromatographed with a solvent composed of *n*-butylacetate, acetic acid, ethanol and water. One of the chromatograms was sprayed with Partridge reagent and the other with Ehrlich reagent. As is shown in Fig. 3A, N-acetylhexosamine, L-fucose and two degradation products of sialic acid[22] were detected in the case of the hydrolysate by 1.5 hours' heating, and galactose besides in the case of the hydrolysate by 3 hours' heating. In order to decide whether the N-acetylhexosamine in the former hydrolysate was the acetyl derivative of glucosamine or galactosamine, two 0.1 cc. portions were taken from the balance of the solution paper-chromatographed above and sampled side by side on a paper sliver. The sliver irrigated as above was cut longitudinally into halves. One of the half-slivers was sprayed with Partridge indicator, and the spot corresponding to N-acetylhexosamine on the other half-sliver was cut out, eluted with water, and after transferring to a small dish, placed in a vacuum desiccator containing $CaCl_2$. The evaporated residue was dissolved in 0.1 cc. of water, and a 0.05 cc. portion was subjected to electrophoresis according to Kunkel and Tiselius,[23] carrying out reference runs with N-acetylglucosamine and N-acetylgalactosamine. The hexosamine in question was identified with glucosamine (Fig. 3B).

The part absorbed on the Dowex of each of the hydrolysates was eluted by displacement with 0.5 *N* NH_3, and the eluate and washing were concentrated to syrupy consistency by vacuum distillation. The residue gave only a spot of aspartic acid, very faint spots of glutamic acid, glycine and alanine excepted, on a chromatogram in the case of the hydrolysate by 1.5 hours' heating (Fig. 3CI), and a spot of leucine and/or isoleucine besides distinct spots of the amino acids above in the case of the hydrolysate by 3 hours' heating (Fig. 3CII).

Oxidation of MP Ia with sodium hypoiodite

The procedure was the same as above. The product (MP Ia') contained

one hexosamine molecule less than the substance before oxidation.

Identification of hexosaminic acid in the hydrolysate of MP Ia'

100 mg. of MP Ia' were taken up in 100 cc. of 1 *N* H_2SO_4 and heated in a boiling water-bath under reflux for 5 hours. The hydrolysate was freed from SO_4-ions by means of baryta, and the filtrate and washing were distilled together in vacuo to about 5 cc. and placed in a vacuum desiccator containing $CaCl_2$. The evaporated residue was dissolved in 0.2 cc. of water, and 0.04 cc. portions were paper-chromatographed with solvents butanol-pyridine-water (5:3:2 by volume) and butanol-acetic acid-water (4:1:2 by volume) and indicated with ninhydrin. Glucosaminic acid was used in a reference run, and MP I treated

FIG. 3A

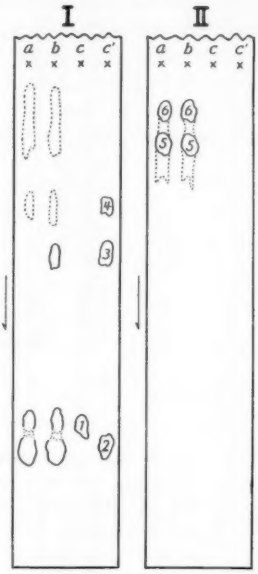


FIG. 3B

Fig. 3A. Chromatograms (2/15×) of the effluent from Dowex 50 of the dialysate of MP I after acetic acid hydrolysis. Developed with *n*-butyl-acetate-acetic acid-ethanol-water (3:2:1:1 by volume) at 24°C for 30 hrs., and indicated with the aniline hydrogen phthalate reagent of Partridge (**I**) and a *p*-dimethylaminobenzaldehyde reagent (Dissolve 0.8 g. of Ehrlich reagent in a mixture of 30 cc. of conc. HCl and 150 cc. of 95% alcohol)[29] (**II**).

a: the effluent after 1.5 hours' hydrolysis. b: the effluent after 3 hours' hydrolysis. c: reference run with N-acetylglucosamine. c': reference run with a mixture of L-fucose, galactose and glucosamine hydrochloride. 1: N-acetylglucosamine, 2: L-fucose, 3: galactose, 4: glucosamine hydrochloride, 5, 6: degradation products of sialic acid.

FIG. 3B. Paper-electrophoretic pattern (1/5×) of the eluate of a spot of N-acetylhexosamine in a similar chromatogram to that in Fig. 3A. The paper spotted with the eluate was sprayed evenly with *M*/20 borax of pH 9.6 until it became translucent, and after fitting in the assemblage of Kunkel and Tiselius, exposed to a 1.5 mA current per cm. width at 500 V for 5 hrs. (Under those conditions N-acetylglucosamine and N-acetylgalactosamine migrated 5.8 and 8.5 cm. respectively towards the anode). Indicator aniline hydrogen phthalate.

a: reference run with N-acetylglucosamine. b: reference run with N-acetylgalactosamine. c: the eluate.

by the hydrolysis procedure above in another. However, the comet-like shaped

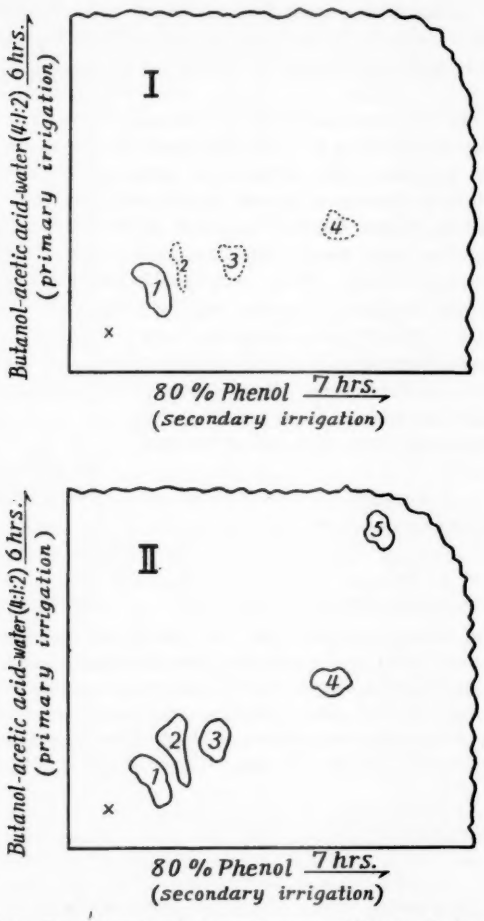


Fig. 3C. Two-dimensional chromatograms (3/15×) of the part absorbed on Dowex 50 of the hydroysates by 1.5 hrs.' (**I**) and 3 hrs.' (**II**) heating with acetic acid of MP I. Temperature of test 24°C. Indicator a 0.1% ninhydrin solution in butanol saturated with water.

1 : aspartic acid, 2 : glutamic acid, 3 : glycine, 4 : alanine, 5 : leucine and/or isoleucine.

spot of glucosaminic acid was of an Rf value similar to those of glutamic and aspartic acids, and hence, the identification was attempted anew by paper electrophoresis in the field of constant 500 V by a 0.9–1.2 mA current per cm. width, applying 0.05 *M* borax as the medium. The assemblage was set up according to Durrum.[24] The test was continued for 4 hours. In the reference runs,

glucosaminic acid migrated 10 cm. towards the anode from the start, glutamic and aspartic acids moved 13 cm. in the same direction, and neutral amino acids and hexonebases remained near the start or moved in the opposite direction. A ninhydrin-positive spot was given at the position corresponding to glucosaminic acid in the main run. It was cut out of the sliver and placed on another sliver wetted with $M/20$ borax plus glacial acetic acid (pH 4.5) to again subject to electrophoresis. The spot behaved like glucosaminic acid here also, migrating 6 cm. towards the anode (Fig. 4).

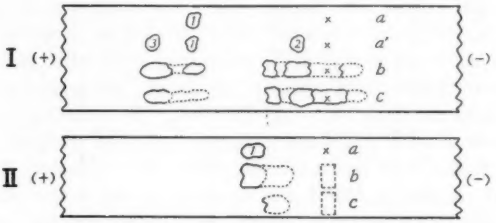


Fig. 4. Patterns in diagram (1/5×) after paper-electrophoresis of the hydrolysate of MP Ia′ in 0.05 M borax (**I**) and the spot at the position of hexosaminic acid in (**I**) in 0.05 M borax plus 30% acetic acid (pH 5.0) (**II**) by a 0.9–1.2 mA current per cm. width at constant 500 V. Duration of test 4 hrs. Indicator 0.2% ninhydrin in glacial acetic acid-ethanol (2:8 by volume).

a: reference run with glucosaminic acid. a′: reference run with a mixture of glucosaminic acid, glutamic acid and alanine. **I**b: hydrolysate of MP Ia′. **I**c: hydrolysate of MP I. **II**b: cut piece from the **I**b-chromatogram, holding the spot of hexosaminic acid. **II**c: cut piece from the **I**c-chromatogram, corresponding to **II**b. (The rectangles surrounded by a broken line are the paper pieces.) 1: glucosaminic acid, 2: alanine, 3: glutamic acid.

A free amino group or groups in MP Ia and I

200 mg. of MP Ia and the same weight of sodium carbonate were dissolved together in 10 cc. of water, and after addition, under vigorous shaking, of 5 cc. of ethanol containing 0.4 cc. of dinitrofluorobenzene (DNFB), further shaken for two hours in ice-water avoiding light, followed by standing overnight in a refrigerator. The reaction mixture was added to with 10 cc. of water and exhausted with 50 cc. portions of ether to completely remove the excess DNFB. Then it was freed from ether by short vacuum distillation and shaken with 3 g. of Amberlite IR-120 for 30 minutes. Filtered. The filtrate and washing united were distilled under reduced pressure to a syrup, precipitated with alcohol and washed with ether. The product dried (DNP-MP Ia) was a yellow powder and weighed 160 mg. In a similar manner, a corresponding product (DNP-MP I) was obtained in a similar yield from 200 mg. of MP I, but this was colored much less.

80 mg. of DNP-MP Ia were hydrolyzed with 8 cc. of 6 N HCl by heating

at 100°C for 12 hours, and the hydrolysate was extracted three times with an equal volume of ether. The ethereal solutions were transferred to a small dish and placed in a vacuum desiccator containing solid caustic soda, and the evaporated residue was further evaporated repeatedly with water to expel off the hydrochloric acid as far as possible. Portions of the final residue were paper-chromatographed with solvents benzylalcohol-ethanol (9:1 by volume) saturated with 0.2 *M* phthalate buffer of pH 6.0[25] and butanol saturated with 3% ammonia.[26] Spots of DNP-glutamic acid were given together with those of dinitrophenol and dinitroaniline (Fig. 5A). To confirm the result, the balance of the final evaporated residue above was heated in a sealed tube with 30% ammonia at 100°C for 6 hours[27], and distilled in vacuo to dryness. The still-residue was taken up in 1 *N* HCl, exhausted with ether, and after removing most of the hydrochloric acid similar to above, examined paper-chromatographically. Glutamic acid only was detected. The ether-extractible fraction of the hydrolysate of DNP-MP I gave no spots of DNP-glutamic acid but only those of dinitrophenol and dinitroaniline (Fig. 5A). Namely the amino-group of glutamic acid residue in MP I was set free by the acetic acid treatment.

Next the HCl-hydrolysates exhausted with ether above were distilled in vacuo to dryness, and each of the residues was dissolved in 10 cc. of 1 *N* HCl and passed through a column of talc (Cf. Sanger[28]) of 2×20 cm. size, followed by washing with 40 cc. of 1 *N* HCl. The effluent and washing were discarded. The part absorbed on the talc was eluted with 50 cc. of a mixture of 4 parts of ethanol and 1 part of 1 *N* HCl by volume, and the yellowish eluate was distilled in vacuo to syrupy consistency and freed from HCl in vacuo over solid caustic soda as above. The final evaporated residue was decomposed with the sat. ammonia and distilled at a low temperature, and the still-residue was subjected to paper chromatography. A spot of lysine appeared in the chromatogram in the both cases of the hydrolysates, confirming that ε-amino group of lysine was dinitrophenylated in MP Ia as well as MP I. (Fig. 5B)

Catalytic hydrolysis of MP I with mercuric chloride

A 200 mg. lot of MP I was dissolved in 10 cc. of a 2% mercuric chloride solution and warmed at 60°C for 30 minutes, whereby a cloudiness occurred. The turbid solution was dialyzed against several changes of water, and the flocculent precipitate was spun down. The clear supernatant, which was perfectly free from mercuric ions (Tested by passing H_2S gas), was distilled in vacuo to a syrup, precipitated with alcohol and after washing with ether, dried in vacuo over $CaCl_2$. The substance recovered (MP Ib) comprised hexosamine, galactose, L-fucose and sialic acid in approximate molecular proportions of 6:5:2:0 (Table IIA), in other words, one molecule each of hexosamine and sialic acid were cleft from MP I by the mercuric chloride treatment, but none of the L-fucose molecules. See also Table IIA, regarding its indirect-Osaki-Turumi reaction.

Detection of sialic acid and N-acetylhexosamine in the dialysate of the solution after treatment with mercuric chloride of MP I

The dialysate above was passed through a 1.5×10 cm. column of Am-

FIG. 5A

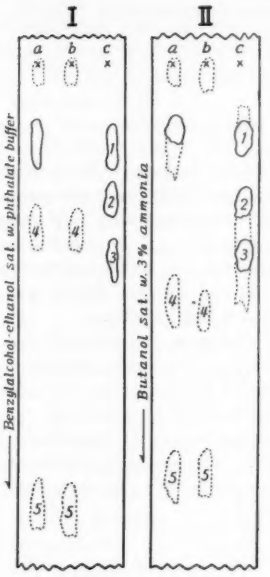


FIG. 5B

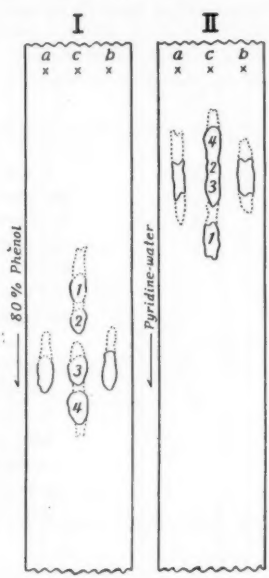


Fig. 5A. Chromatograms (1/5×) of the ether-soluble fraction of the hydrolysates of DNP-MP Ia and DNP-MP I. (**I**) developed with the benzyl-alcohol-ethanol mixture saturated with a phthalate buffer. (**II**) developed with butanol saturated with 3% NH_3. The solvent fronts run 45 cm. Temperature of test 24°C.

a : DNP-MP Ia. b : DNP-MP I. c : reference run. 1 : DNP-glutamic acid, 2 : DNP-glycine, 3 : DNP-glucosamine, 4 : dinitrophenol, 5 : dinitroaniline.

Fig. 5B. Chromatograms (1/5×) of the fractions of DNP-MP Ia and of DNP-MP I, eluted from the talc and decomposed with ammonia. Irrigation was effected with 80% phenol (A dish containing 3% ammonia was placed in the cylinder) (**I**) and with pyridine-water (65:35 by volume)[30] (**II**). Temperature and duration of test 24°C and 20 hrs. respectively. Indicator 0.1% ninhydrin in butanol saturated with water.

a : the fraction of DNP MP Ia. b : the fraction of DNP-MP I. c : reference run. 1 : histidine (grey), 2 : ornithine (reddish purple), 3 : lysine (purple), 4 : arginine (purple).

(The ornithine specimen was kindly given by Dr. K. Shimura in the Department of Argicultural Chemistry, which is appreciated.)

berlite IR-120, followed by energetical washing of the column with 100 cc. of water, and the strongly acidic effluent and washing were passed through a column (1.5×10 cm.) of Amberlite IR-4B (carbonate-form). The effluent and washing here were distilled together in vacuo to a small volume and placed in a de-

siccator. The syrupy residue was paper-chromatographed. The presence of N-acetylhexosamine and sialic acid were demonstrated (Fig. 6).

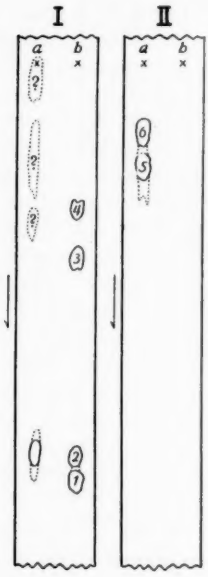


Fig. 6. Chromatograms (2/15×) of the dialysate after hydrolysis of MP I with mercuric chloride, developed by irrigation with *n*-butylacetate acetic acid-ethanol-water (3:2:1:1 by volume) for 30 hrs. at 24°C. Indicated with aniline hydrogen phthalate of Partridge (**I**) and with the Ehrlich reagent (See the explanation of Fig. 3A) (**II**).

a: the dialysate. b: reference run with a mixture of L-fucose, acetylglucosamine, galactose and glucosamine hydrochloride. 1: L-fucose, 2: N-acetylglucosamine, 3: galactose, 4: glucosamine hydrochloride, 5, 6: degradation products of sialic acid. (The spot of N-acetylhexosamine is colored characteristically greyish brown by Partridge reagent.)

Summary

1. A group mucopolysaccharide preparation from pig stomach mucus, which had the minimum molecular weight of 3500 as calculated from its iodine use, contained approximately seven molecules of hexosamine, five molecules of galactose, two molecules of L-fucose, one molecule each of sialic acid, aspartic and glutamic acids, serine and alanine and amounts lesser than one molecule of various amino acids for a unit molecule corresponding to the minimum molecular weight.

2. The group mucopolysaccharide was subjected to oxidation with hypoiodite, hydrolysis with acetic acid and treatment with mercuric chloride, and the partially degraded product by acetic acid also to the treatment with hypoiodite. And the products were examined scrupulously.

3. The structure of the reducing residue of the carbohydrate moiety and its neighbourhood in the unit molecule is suggested.

The expenses of this work were defrayed by a grant from the Ministry of Education through the Grant Committee for Scientific Researches, which is gratefully acknowledged. H. Masamune.

References and Notes

1) Masamune, Nihon Seikagaku Kaishi (Transactions Jap. Bioch. Soc.), 1944, **18**, 247; Masamune, Tohoku J. Exp. Med., 1949, **50**, 107; Masamune, Yosizawa & Maki, *ibid.*, 1951, **53**, 237 (Footnote on p. 237); Masamune, Seitai no Kagaku (Science of Living Organisms), 1952, **3**, 234 (Explanation of Fig. 1).

2) Masamune, Nihon Seikagaku Kaishi (Transactions Jap. Bioch. Soc.), 1944, **18**, 247.

3) Stacey & Woolley, J. Chem. Soc. London, 1942, 550.

4) Werner, Acta soc. med. Upsal., 1953, LVIII, 1.

5) Masamune, Tohoku J. Exp. Med., 1953, **57**, 310.

6) Hakomori, *ibid.*, 1954, **60**, 331.

7) Vunakis & Kabat, J. Am. Chem. Soc., 1951, **73**, 2977.

8) Partridge & Davis, Nature, 1950, **165**, 62.

9) Aminoff & Morgan, Bioch. J., 1951, **48**, 74.

10) Masamune & Yosizawa, Tohoku J. Exp. Med., 1954, **60**, 135.

11) Sanger, Bioch. J., 1945, **30**, 507.

11a) Kuhn & Kirschenlohr, Chem. Ber., 1954, **87**, 1547.

12) Masamune & Yosizawa, Tohoku J. Exp. Med., this volume, p. 53.

13) Yosizawa, *ibid.*, 1951, **55**, 35.

14) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.

15) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.

16) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.

17) Masamune, H., Hakomori & Masamune, O., Tohoku J. Exp. Med., 1954, **60**, 347; Masamune & Hakomori, *ibid.*, this volume, p. 59.

18) Macleod & Robison, Bioch. J., 1929, **23**, 517.

19) Yosizawa, Tohoku J. Exp. Med., 1949, **51**, 51.

20) Masamune, H., Hakomori, Masamune, O. & Takase, *ibid.*, this volume p. 67.

21) Masamune, Hakomori, Numabe, Akama & Kamiyama, *ibid.*, 1955, **61**, 283 (ff. 294–295).

22) In addition, two purple spots of Rf 0.1 and 0.08 were shown when the same solution was paper-chromatographed with solvent butanol-pyridine-water (5:3:2 by volume) and indicated with Ehrlich reagent. Klenk and Faillard (Z. physiol. Chem., 1954, **298**, 230) said already that sialic acid decomposes on standing its watery solution and gives spots of low Rf values.

23) Kunkel & Tiselius, J. Gen. Physiol., 1951, **35**, 89.

24) Durrum, J. Am. Chem. Soc., 1950, **72**, 2943.

25) Blackburn & Lowther, Bioch. J. 1951, **48**, 477.

26) Mellon & Hoover, J. Am. Chem. Soc., 1953, **75**, 1675.

27) Lowther, Nature, 1951, **167**, 767.

28) Sanger, Bioch. J., 1949, **45**, 563.

29) Hamasato & Akakura, J. Bioch. (Japan), 1941, **34**, 159.

30) Bentley & Whitehead, Bioch. J., 1950, **46**, 341.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

The Sodium-Potassium Ratio in Saliva of Children with or without Nutritional Dystrophy

(Studies on the Nutrition of Children in Hirosaki Area)
(38th Report)

By

Yoshinori Ishioka
(石岡 好憲)

From the Department of Pedicatrics, School of Medicine, Hirosaki University, Hirosaki. Director: Prof. Ts. Arakawa

(Received for publication November 12, 1955)

As will be seen from a preceding paper from our Laboratory,[1] an abnormal response to Thorn's test was found in 5 cases out of 18 cases of the children with nutritional dystrophy. In 1952 Grad[2] reported that the sodium-potassium ratio in saliva might be a good index representing the adrenocortical salt-regulating activity. In the present paper the adrenocortical activity of children with nutritional dystrophy[3),4)] will be discussed from the view point of the sodium-potassium ratio in saliva of them.

Experimental

Method and Material

Samples of unstimulated mixed saliva[2] are obtained at 11 a. m., centrifuged, and aliquots of upper clear layer are used for estimation of sodium and potassium. For flame analysis of sodium and of potassium, Shimadzu-flame-photometer (Type Q B-50) was used, and dilution of saliva made up to 1 :20 and 1 :40 for estimation of sodium and for that of potassium respectively.

The estimation of sodium and of potassium in saliva was made on 29 boys from 8 to 10 years of age. These boys were divided into 2 groups (Groups I and II). Group I includes 15 boys without nutritional dystrophy, and Group II 14 boys with nutritional dystrophy.

Results and Comment

The mean values of the sodium-potassium ratio in saliva were 0.57 ± 0.04 and 0.48 ± 0.06 in Groups I and II respectively. (Cf. Tables I and II).

So the mean value of the sodium-potassium ratio in saliva tends to

TABLE I

Sodium and Potassium Concentration in Saliva of Group I

No. of case	Na mEq/L	K mEq/L	Na/K
1	14.	20.0	0.71
2	13.8	20.5	0.67
3	11.2	19.7	0.57
4	8.1	13.8	0.59
5	8.8	15.7	0.56
6	8.8	16.1	0.55
7	9.8	16.2	0.60
8	9.0	16.7	0.54
9	10.0	19.7	0.51
10	9.8	16.0	0.61
11	10.1	17.0	0.59
12	7.9	18.6	0.42
13	12.3	23.5	0.52
14	9.6	16.0	0.60
15	8.9	15.5	0.57
	Average		0.57±0.04

TABLE II

Sodium and Potassium Concentration in Saliva of Group II

No. of case	Na mEq/L	K mEq/L	Na/K
1	9.5	21.3	0.45
2	12.1	18.5	0.65
3	8.6	17.3	0.50
4	10.8	23.7	0.46
5	12.0	21.7	0.55
6	8.2	22.2	0.37
7	8.9	18.6	0.48
8	11.2	18.6	0.60
9	8.8	20.6	0.43
10	11.5	20·6	0.56
11	8.4	15.9	0.53
12	7.4	21.7	0.34
13	11.2	21.8	0.51
14	7.8	23.4	0.33
	Average		0.48±0.06

be lower in the boys with nutritional dystrophy than in those without nutritional dystrophy.

The sodium-potassium ratio in the unstimulated mixed saliva of normal persons ranges from 0.2 to 0.6, according to Grad[5] who used flame analysis. Our results show a fairly good accordance with those of Grad.[5] Hoffmeister *et al.*,[6] using chemical methods for the estimation of these minerals in saliva, reported that the normal mean value of the sodium-potassium ratio in saliva which was collected by stimulation with paraffine-chewing was 0.59 ± 0.04.

Thus it will be seen that a fall or a rise in the sodium-potassium ratio in saliva is taken to indicate an increase or a decrease in adrenal cortical salt regulating activity, whether it is the unstimulated mixed[2] saliva or the saliva collected by stimulation with paraffine-chewing.[7]

As regards the relation between dietary deficiency and endocrine glands, McCarrison[8] reported that the adrenal glands were hypertrophied and that the adrenalin content was high when pigeons and monkeys were fed on the diet which was deficient in B vitamine, suitable in protein and disproportionately rich in starch. In 1950, Skelton[9] showed that, on the experimentation of rats, B-complex deficiency, per se, caused adrenal hypertrophy and thymus atrophy and that it must be considered as a non-specific stress.

On autopsy of pellagrins, atrophy of the adrenals was reported by Susman[10] and Thannhauser.[11] According to Jackson,[12] however, atrophy of the adrenals could not be anticipated consistently in chronic malnutrition and in pellagra.

Gillman and Gillman[13] described that in the early stage of malnutrition and of pellagra, there might be no visible structural changes in the adrenals, while later the glands, they stated, might be hypertrophied and subsequently undergo atrophy.

The majority of the boys who were included in Groups II in the present investigation must be taken to be in a state of nutritional dystrophy, because, apparently healthy as they were, they had the following items: ariboflavinotic signs,[3] low level of riboflavin in blood[14] and in urine,[15] pellagrous skin lesions,[3] a decrease in urinary excretion of N′-methylnicotinamide,[11] macrocytic anemia,[3),17] a decrease in protein bound iodine in serum,[18] a decrease in albumin and an increase in globulins in electrophoretic pattern of serum[19] and histopathological lesions of the biopsied livers.[20]

The explanation for low value of the sodium-potassium ratio in saliva of these malnourished children may be given in one of the following two ways:—Firstly, it results from hyperactivity of the adrenals, which develops as a precursive stage to atrophy of the adrenals in malnutrition,[13] or which is caused by a factor or factors[21] other than deficiency in nutrients in malnutrition. Secondarily, it is explained by the relation of hepatic

lesions to the adrenal hormones. According to Bongiovanni *et al.*[22] and Bruckel *et al.*,[23] adrenal cortical hormones are inactivated in the liver, and urinary output of corticoids is increased in acute hepatitis and in Laennec's cirrhosis. Sato,[20] in our Laboratory, confirmed histopathological alterations in the biopsied liver specimens from the children with nutritional dystrophy. It is therefore suspected that an inactivation of adrenal cortical hormone is decreased in the livers of children with nutritional dystrophy.

Summary and Conclusions

The mean value of the sodium-potassium ratio of the unstimulated mixed saliva is slightly lower in boys with nutritional dystrophy than in those without it. A rise in salt regulating activity of the adrenals of the boys with nutritional dystrophy is suggested.

This work was carried out by a Grant in aid for Scientific Research of the Ministry of Education. Prof. Ts. Arakawa.

References

1) Sasaki & Hashimoto, Hirosaki Igaku (Japan), 1953, **4**, 39.
2) Grand, J. Clin. Endocrinol., 1951, **11**, 152.
3) Masuda, & Aoyama, Tohoku J. Exp. Med., 1951, **55**, 1.
4) Arakawa, Wada, & Takahashi, *ibid.*, 1951, **55**, 61.
5) Grad, J. Clin, Endocrinol., 1952, **12**, 708.
6) Hoffmeister, & Albrecht, Klin, Wschr., 1953, **31**, 567.
7) Frawley, & Thorn, Proc. 2. Clin. ACTH Conf., 1951, **1**, 115. (Cited from 80.
8) McCarrison. Brit. Med. J., 1920, **11**, 236.
9) Skelton, Am. J. Physiol., 1950, **161**, 515.
10) Susman, Edinburg M. J., 1926, **33**, 58. Cited from 14.
11) Thannhauser, Munch. Med. Wschr., 1933, **80**, 219.
12) Jackson, The effects of inanition & malnutrition upon growth & structure, Blakiston's Son & Co., Philadelphia, 1925, Cited from 14.
13) Gillman, & Gillman, Perspectives in human malnutrition, Grune & Stratton, New York 1951.
14) Kagaya, Vitamins (Japan), 1954, **7**, 557.
15) Kagaya, Riboflavine Concentration of Urine from Children with or without Nutritional Dystrophy. To be published in a coming issue of Vitamins.
16) Masuda, Vitamins (Japan), 1953, **6**, 187.
17) Wada, & Arakawa, Tohoku J. Exp. Med., 1952, **56**, 47.
18) Hashimoto, Protein-bound-iodine Level in Serum of Children with Nutritional Dystrophy. In press in this Journal.
19) Yoshida, Tohoku J. Exp. Med., 1953, **57**, 205.
20) Sato, Liver Biopsy Findings of Children with Nutritional Dystrophy. In press in this Journal.
21) Schettler, Klin. Wschr., 1952, **30**, 229.
22) Bongiovanni, & Eisenmenger, J. Clin. Endocrinol., 1951, **11**, 152.
23) Bruckel, Hubner, Meyerheim, & Liesrsch, Klin. Wschr., 1954, **32**, 21.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

The Effects of Glucuronic Acid on Acne Vulgaris

By

Susumu Tsukada, Tsunao Tanida, Atsunobu Aikawa
(塚田 進) (谷田津奈男) (愛川篤信)
and **Junichiro Mihara**
(三原潤一郎)

From the Department of Dermatology; Director: Prof. M. Ito and the Department of Dermatology, Nagamachi Branch Hospital; Director: Asst. Prof. S. Tsukada, Faculty of Medicine, Tohoku University, Sendai

(Received for publication, November 16, 1955)

One of our colleagues, S. Kikuchi, has conducted the intravenous glucose tolerance test in patients with various skin diseases as will be published in the Jap. J. of Dermat. in the near future and found that pyodermas, generally speaking, in 41.7% cases showed intolerance to sugar, of which both carbuncles and furuncles in 36.8% and acne vulgaris in 50% showed intolerance to sugar. The pathoglucemic curve obtained in this study is not understood to be prediabetic curve, but as a curve showing disturbance in liver function (Soskin & Levine[1]). The acne vulgaris cases that have come under our treatment are 27 in number (6 males and 21 females) and intolerance to sugar are observed in 55.6% of them. Skin sugar was measured in only one of the above, and was recorded as 89 mg/100 g in fasting value, while his blood sugar in fasting showed the value of 98 mg/dl, so that the ratio of skin sugar/blood sugar stood at 90.8%. These figures are not abnormal as compared with the normal values of 89.8 mg/100 g of skin sugar and 89.8% of the ratio between skin and blood sugar in normal Japanese, obtained by Mihara (Fasting value of skin sugar differs from that of Urbach *et al.*[2] " Studies on Skin Sugar in Normal Japanese and Skin Diseases " by Mihara will shortly appear in the Jap. J. of Dermat.). The time of recovery of the starting value of skin sugar following a glucose tolerance test, however, was 226 minutes in this case, a value much in excess of the normal 60 minutes. The recovery time of blood sugar was 38 minutes, a value well within the normal range. From these results, we may conclude that the skin of this patient retains sugar in it for a long time. We must wait for test results with many further cases to speak with some confidence whether such a sugar retention in the skin in acne vulgaris patients is of common occurrence, but this single case suggests something of interest, in con-

nection with infection of this disease. Among the 27 cases tested, 74% showed positive reaction in urobilinogen test, 67% in Takata's reaction, and 44.4% in BSP. 56% showed familiar occurrence, 23.8% menstrual anomalies and 47.6% premenstrual exacerbation. Now, as the pathogenic causes of acne vulgaris, we are used to think of predisposition to it and unbalance of sex hormones, seeking them chiefly in anomalies of the ratio between androgen and estrogen or between androgen plus progesterone and estrogen (Wile, Snow & Brandberg,[3] Lawrence & Werthessen,[4] Hamilton[5]). Besides these, dietary deficiency, vitamin dificiency, infection and mode of life are looked upon as the main secondary factors. Thus, the principle of treatment of this condition has been laid in the adjustment of A/E or A+P/E ratio (Aron-Brunitière[6]), consisting in administration of estrogens (Lawrence & Werthessen[4], Goeckerman,[7] Goeckerman & Wilhelm,[8] Sawicky, Danto & Maddin,[9] Jarrett,[10] Yasuda[11]), or since gonadotropin (Aron-Brunitière[6]) in small dosis tends to stimulate FSH and in large dosis to stimulate LH, in administration of a small dosis of gonadotropin and in fact, good results have been obtained thereby. The method of treatment of the patients with acne vulgaris adopted by the authors of this paper consists in oral administration of Guronsan, a glucuronic acid preparation, in doses of 3 gm. 3 times daily and parenteral administration of 400 mg. of it simultaneously. This therapy usually takes effect within 1–3 months and is efficacious whatever the type of acne vulgaris of the case. A marked decrease in seborrhoea is the first effect to be noted in this therapy. The method has shown cure in 52% and highly satisfactory results in 26% of the treated cases, showing a total percentage of successful cases of 78%. And no one case showed aggravation of the disease in the course of this treatment. In 6 cases, we could not follow up the subsequent course of the disease. Upon following up the far results of cases of success several months after the treatment, we got information that light relapse was observed in 26.3% cases among the total inquired, but these cases of relapse suffered only in imcomparably slighter degree than in the original affliction. In such cases of relapse, we are using Guronsan another time, and irradiation of ultraviolet light is applied simultaneously.

As a few findings of special interest upon Guronsan administration, we may point to the relief from obstipation, so prevalent among acne vulgaris cases, experienced by some of our cases, and the recovered regularity of irregular menstruation or cure from dysmenorrhoea in female patients. These findings may be explained as natural, if we are justified in our interpretation of the efficacy of glucuronic acid against acne vulgaris. In conclusion of this paper, we will give the following interpretation on the efficacy of glucuronic acid upon acne vulgaris. Now, it is known

that steroid hormones are detoxicated or inactivated in the liver, that is, the liver has the function of regulating the amount of these sex hormones, for maintaining the ratio A/E or A+P/E within the physiological range. The principal role in this regulative activity is borne by glucuronic acid. When an abnormality in the above ratio has been brought about, the rate of gonadotropin secretion from the anterior lobe of the pituitary also falls into abnormality, this again expediting the abnormality in the A/E or A+P/E ratio in a vicious circle. If then, this vicious circle is cut in the liver and the ratio of sex hormones is restored to normality, the rate of gonadotropin secretion by the anterior pituitary gland answering the abnormality of the said ratio is normalized, thus establishing a normal ratio between the steroid hormones and gonadotropin. Such is our opinion on the action of glucuronic acid, represented in our study by Guronsan. The aim of therapy for acne vulgaris, whether by gonadotropin or by Guronsan, must be in the final analysis in normalizing the ratio of the sex hormones and thereby in adjusting the secretion of gonadotropin. The Guronsan therapy we have tried may be called a specific treatment for acne vulgaris, because it meets this requirement admirably, and moreover, is lasting in its effect.

Summary

1. The liver function tests revealed that the majority of patients with acne vulgaris showed the disturbance of liver function.

2. It was shown that the glucuronic acid administration yielded remarkably beneficial effects upon acne vulgaris.

3. The authors discussed why glucuronic acid highly efficacious on acne vulgaris was.

References

1) Soskin & Levine, Carbohydrate Metabolism, Chicago, the University of Chicago Press, 1947.
2) Urbach & Lentz, Arch. Dermat. & Syph., 1945, **52**, 301.
3) Wile, Snow & Brandberg, *ibid.*, 1939, **39**, 200.
4) Lawrence & Werthessen, N. T. Endocrinology, 1940, **27**, 755.
5) Hamilton, J. Clin. Endocrinol., 1941, **1**, 570.
6) Aron-Brunetière, Brit. J. Dermat., 1953, **65**, 157.
7) Goeckerman, Arch. Dermat. & Syph., 1950, **61**, 237.
8) Goeckerman & Wilhelm, *ibid.*, 1952, **66**, 402.
9) Sawicky, Danto & Maddin, *ibid.*, 1953, **68**, 17.
10) Jarrett, Brit. J. Dermat., 1955, **67**, 165.
11) Yasuda, Clin. Endocrinol., 1955, **3**, 65.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Neutralization and Propagation of Retinal Induction in Human Eye

By

Dairin Nakagawa and **Tadashi Kohata**
(中川大倫) (木幡正)

From the physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, December 16, 1955)

Introduction

The phenomenon of retinal induction discovered by Motokawa was shown to be a retinal process underlying contrast[1] and other psychological phenomena.[2-5] This phenomenon was found not only in the human eye, but also in mammalian retinas,[6] and further it was shown that retinal induction is caused not only by colored light, but also by intermittent white light[7] and by alternating currents.[8] By means of a new phenomenon, neutralization of retinal induction, Motokawa[1-3] made a series of experiments which suggest the propagation of the induction effect in the retina, and Motokawa, Iwama & Ebe[9] measured the velocity of propagation in the retinas of man and cats. Moreover, Motokawa and Ebe[10] showed that pre-established retinal induction could not be abolished by antidromic stimulation of the optic nerve.

The present experiment was undertaken to clarify the mechanism of neutralization and propagation of retinal induction.

Experimental

Method

The method used was essentially the same as that employed by Motokawa. It consists in measuring the electrical sensitivity of the eye after exposure to light. The stimulating current used was a constant current pulse of 100 msec. It was applied to the subject's eye through a pair of silver electrodes of 2×1.5 cm^2 in size, one of which was placed above the eyebrow and the other on the homolateral temple of the subject.

The effect of light measured with electrical phosphenes as an index, was expressed in terms of ζ which was defined by the formula: $\zeta = 100 (E-E_o)/E_o$, where E and E_o denoted electrical excitabilities (reciprocals of threshold) measured with and without pre-illumination.

The retinal induction effect is determined by the difference between the ζ value for white light alone and the ζ value for white light that has been preceded by colored light, and denominated the contrast effect (C. E.).

The spectral lights used in the present experiment were adjusted to equal energy. Colored lights from color-filters were sometimes used in addition to spectral light. Their intensities were given in terms of the illumination measured by a luxmeter on the stimulating surface without the filter. The white test-light used was always 40 lux in intensity, and its size 0.4° in visual angle.

The patch was illuminated from behind, and the distance between the patch and the subject's eye was always 30 cm. The left eye of the subject was always used, while the right eye was shielded. All measurements were performed after dark adaptation of about 20 minutes. The optical arrangements and procedures will be mentioned together with the results.

As to the details of the method and precautions to determine the electrical excitability Motokawa's[1-3),11),12)] papers should be consulted.

Results

Neutralization of direct induction

At first, the neutralizing effects of spectral light of various wave-lengths upon the direct induction caused by red or yellow filtered light, were investigated.

A red or yellow light and a white test-light were presented in the same retinal area at an interval of 2 seconds, and the electrical excitability was measured after 2.5 (red) or 3.0 (yellow) seconds from the end of the white test-light.

The results are shown in Fig. 1. As can be seen in this figure, the curve connecting empty circles has a conspicuous reduction of contrast effect at the wave-length 490 mμ complementary to red light. This means that the direct induction set up in the retina by red light, is neutralized only with the spectral lights within a very narrow range having its center at 490 mμ. Data obtained with yellow light as inducing light are represented by empty triangles in the same figure. When compared with the effective range in the former experiment, the range for neutralization of the direct induction caused by yellow light is decidedly wide. The fact that the range is wider for yellow inducing light than for red light cannot be ascribed to the circumstance that the filtered yellow light used in this experiment was less pure than the red light, because the same relation is obtained with monochromatic light.

Next, neutralization of direct induction was investigated as a function

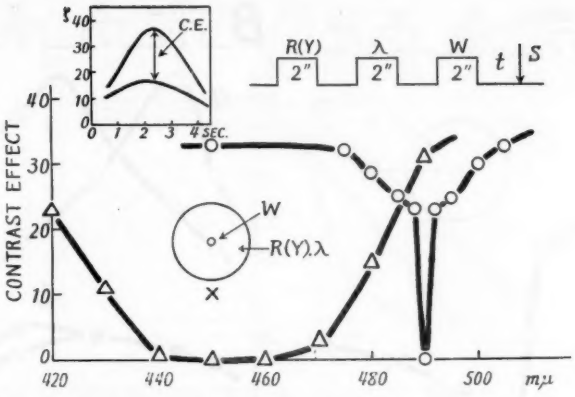


Fig. 1. Neutralization of direct induction.

Ordinate: contrast effects (C. E.), quantity indicated by vertical line in inset. For curve marked by open circles, inducing light was red light (R) and for that marked by triangles, it was yellow light (Y). Intensity of inducing light is about 3 lux without filter. Size of patch is 2°. Cross shows fixation point. Order of application of stimuli is shown in inset.

of the intensity of neutralizing light. In this experiment the red filtered light was used as the neutralizing light, and the spectral light of 490 mμ as the inducing light. The electrical sensitivity was measured 1 second after the end of the white test stimulus. In Fig. 2 (A) contrast effects (C. E.) are plotted against the logarithm of the intensity of the neutralizing light. C. E. values are reduced as the intensity is increased, and become zero at a certain intensity (complete neutralization). It is, however, curious that C. E. values begin to rise again when the intensity is increased further. This must be an apparent increase due to over-neutralization which was described by Motokawa.[4] In order to confirm that this is really due to the over-neutralization, the time course of electrical excitablity was measured under each experimental condition. Data are shown in Fig. 2 (B). Curve, a, having the maximum at 1 second is the ζ-time curve for the direct induction (490 mμ) unaffected by the neutralizing light (R). At the intensity of neutralizing light at which complete neutralization takes place, curve b is obtained. When the intensity of neutralizing light is too high a curve having a peak at about 2.5 seconds is obtained (curve c), and this is obviously, in character, the same as the curve for the direct induction set up by the red light. It is apparent that over-neutralization occurs if the intensity of the neutralizing light is raised too high.

Effect of strong white light upon direct induction

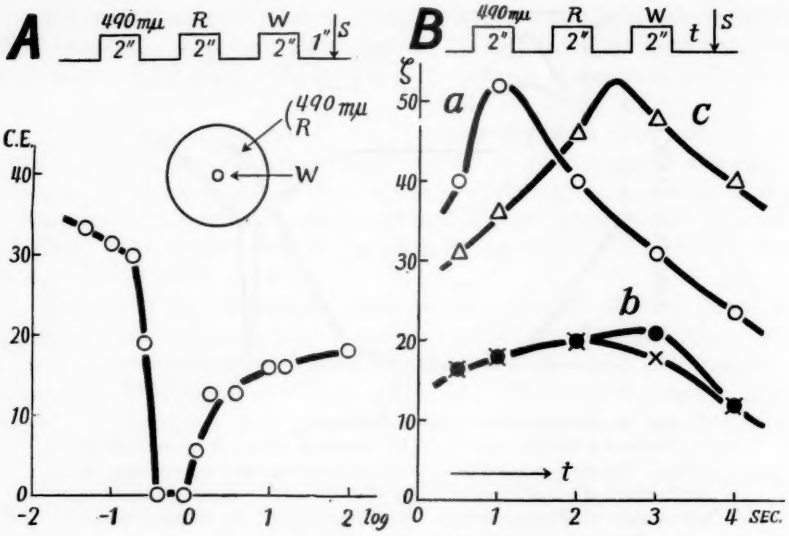


Fig. 2. (A) Neutralization of direct induction as function of intensity of neutralizing light.

Abscissa: log intensity of neutralizing red filtered light (R).

(B) Neutralization of direct induction and over-neutralization.

Ordinate: percentage increases of electrical excitability of retina. Abscissa: time in sec. after removal of white test-light. Excitability curve for white test-light alone is marked by crosses. For curves a, b and c neutralizing red light was too weak, just sufficiently strong for neutralization, and too strong, respectively.

The question now arises as to whether or not the direct induction caused by any spectral light might be modified by strong white light interposed between the inducing light and the white test-light.

The results of the experiments concerning this question are shown in Fig. 3. All curves in the figure indicate that the direct induction set up by spectral lights could not be modified even by very strong white light ranging from 2000 to 5000 lux. But, when white light of higher intensities was applied, it was found effective in reducing C. E. values. It is to be noted that direct induction set up by any colored light is neutralized by its complementary color of extremely low intensity, whereas it is not affected by white light of very high intensity.

In the following experiment the time course of electrical excitability was measured, to get information about its possible effect upon the quality of induction which manifests itself in the time course of excitability. As is shown in Fig. 4, the maximum of excitability appeared always at 2.5

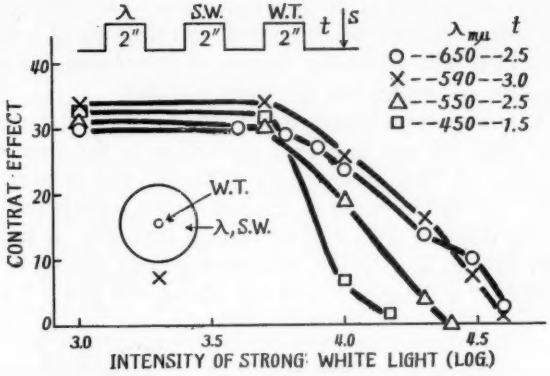


Fig. 3. Effect of strong white light upon direct induction.
Abscissa: log intensity of strong white light applied prior to white test-light.

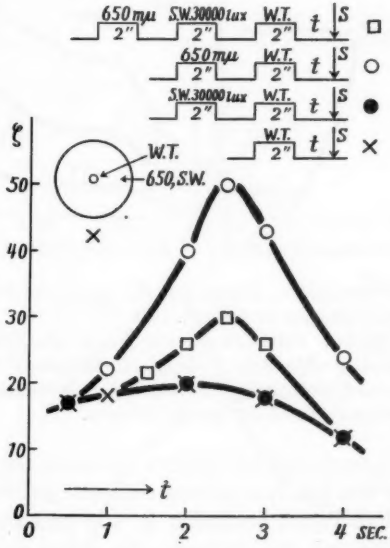


Fig. 4. ζ-time curves affected by strong white light applied prior to white test-light.
Effect consists in reduction of ordinate. No change in form of curve can be seen.

seconds, even when the ordinate of the maximum was reduced by strong white light, and this fact indicates that the quality of induction remains

unaltered under the action of strong white light. The effect of white light upon retinal induction may be compared with dilution of a dye with destilled water which will affect the degree of saturation, but not the hue.

Neutralization and propagation of indirect induction

In the following experiments the indirect induction caused by a red patch, R, at point W was measured according to the procedure for indirect induction. A blue-green patch (490 mμ) was presented at a distance from R. As the intensity of the blue-green light is raised the C. E. value begins to decrease at a certain critical intensity, as indicated by a double arrow in Fig. 5 (A). In this arrangement the set of patches, R and W, is called " a detector " and the blue-green patch " an inducer."

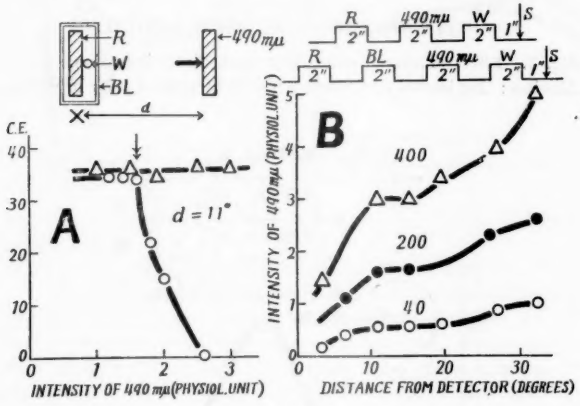


Fig. 5. Measurement of critical intensity of inducer just required to neutralize indirect induction at detector.

Inducer: 490 mμ. Detector: R, red filtered light (200 lux, without filter). BL: blocking white light (10 lux). Size of inducer and detector is 2×20 mm^2 respectively. Intensity of inducer is given in terms of physiological units. One unit equals threshold intensity at fovea.

The threshold intensity of the inducer at which the indirect induction around the detector was just neutralized could be determined in this way. When a white outline figure enclosing R was presented in the interval between the detector and the inducer, the other procedure being the same as above, no neutralization of indirect induction took place, as is shown by the curve connecting triangles. This is because the spreading induction from the inducer is blocked at the blocking white patch (BL), and cannot neutralize the indirect induction around R.

Next, the critical intensity of the inducer was determined at various distances separating the detector and the inducer. The data obtained

are shown in Fig. 5 (B). As the inducer was moved farther from the detector, the critical intensity increased. It is without saying that as the intensity of the detector is higher the critical intensity of the inducer is higher (cf. three curves), but it deserves attention that all three curves have a depression at a certain distance, 15° in visual angle, from the detector. This phenomenon seemed to depend upon the uneven properties of the retina concerning induction, as suggested by the following experiments. The blue-green patch of a fixed intensity was presented to various parts of the retina, and its effect at each position was measured by the method of direct induction. The data obtained are represented by the curve b in Fig. 6 (A). In this curve is seen a striking peak at about 15° from the fovea, and this result resembles the finding by Kurosawa and Katayama.[13] This fact suggests that the capacity to produce retinal induction is greatest at the parafoveal area 15° from the center. Instead of using the blue-green patch of a constant intensity, the critical intensity at each point was used to make a similar experiment. The result is represented by curve a in Fig. 6 (A). As can be seen in this curve, the direct induction caused by the blue-green light of the critical intensity

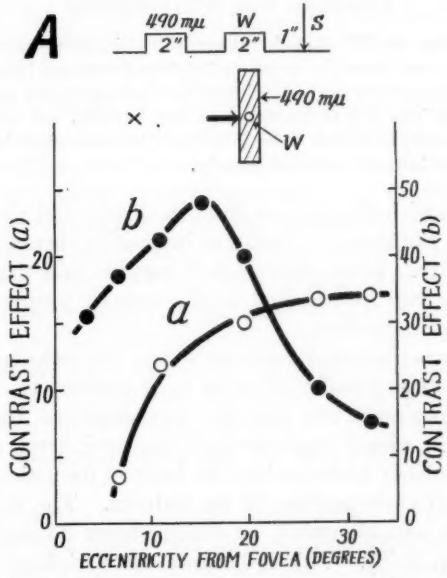


Fig. 6. (A) Direct induction set up at each retinal area by inducer of constant intensity (curve b) and of critical intensity obtained above (curve a).

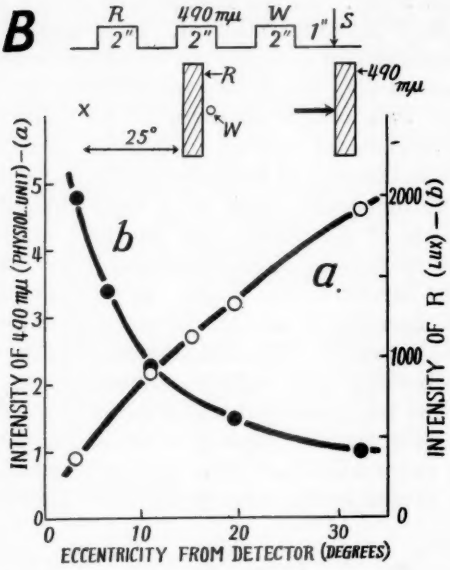


Fig. 6. (B) Experiment carried out in periphery of retina. Detector was located in periphery 25° from fovea, and inducer farther away. Curve a represents critical intensity of inducer at each point to neutralize indirect induction at detector of constant intensity, and curve b refers to critical intensity of detector whose induction is just neutralized by spreading induction from inducer of constant intensity.

increased steadily with the distance from the fovea, and no such depression as was found in the curve for critical intensities themselves is found at 15° from the center. From these facts it may be said that the depression referred to above can be ascribed to the uneven property of the retina concerning retinal induction.

In all the experiments mentioned above the detector was presented to the fovea, although parafoveal areas were exposed to the inducer. In the following experiment the detector was displaced together with the inducer by 25° in visual angle toward the periphery, and the critical intensity of the inducer to neutralize the indirect induction at the detector was determined at each position of the inducer. The data obtained are represented by a smooth curve, a, which shows no such depression as was found in Fig. 5 (B). This is because the periphery of the retina is homogeneous concerning retinal induction as compared with the parafoveal region.

Next, the intensity of the inducer was fixed and that of the detector

was so adjusted that complete neutralization might occur at the detector. As is shown by curve b in Fig. 6 (B), the intensity of the detector decreases as the inducer moves farther away from the detector. This fact indicates that the spreading induction initiated by the inducer suffers from decrement as it is propagated through the retina.

Discussion

Retinal induction, direct or indirect, survives the photic stimulus for about 40 seconds, but disappears promptly when the inducing colored light is followed by the complementary colored light. The present experiment has shown that the range of wave-lengths to neutralize the induction caused by red light is much narrower than that to neutralize the induction caused by yellow light. This difference was not to be ascribed to the difference in the broadness of the ranges of transmission of the filters used to produce the red and the yellow light, because the same result can be obtained when monochromatic lights are used as inducing lights. The complementarity between red and blue-green may, therefore, be regarded as more rigid than that between yellow and blue. For this reason, the pair of red and blue-green is superior to the pair of yellow and blue for detailed measurements of neutralization, but the latter pair is preferable when filters of broad transmission ranges are used both for inducing and neutralizing lights.

White light is ineffective for modifying pre-established retinal induction, but it reduces retinal induction at extremely high intensities. The question now arises as to whether the reduction of retinal induction caused by the white light might be neutralization, because the white light used was the usual incandescent light and somewhat yellowish. In view of the fact that white light of sufficiently high intensity can reduce all sorts of retinal induction caused by various colored lights, as is shown in Fig. 3, the phenomenon under consideration cannot be ascribed to neutralization. We know that extremely unsaturated colored light is incapable of producing retinal induction. The effect of white light may, therefore, be regarded as a kind of desaturation which would be caused in the retina by the white light delivered subsequently. The fact that no effect corresponding to over-neutralization can be observed supports this interpretation.

Our experiments concerning propagated induction suggest that induction suffers from decrement as it is propagated through the retina. The curves a and b in Fig. 6 (B) will give some information about the decrement of energy involved. Further investigation will, however, be required to elucidate the mechanism of this phenomenon.

SUMMARY

The neutralization of retinal induction was quantitatively investigated.

1. The direct induction caused by red filtered light was found to be neutralized by blue-green light from a very narrow range of the spectrum. The range of the spectrum effective to neutralize the direct induction caused by yellow filtered light was found much wider.

2. White light of intensity lower than 5000 lux, which was interposed between the inducing colored light and the white test-light did not affect the direct induction caused by the colored light. At higher intensities of white light the direct induction was reduced, but this effect could not be identified with the neutralization of retinal induction.

3. It was confirmed that the white light stimulus, even when its intensity was raised to 30,000 lux, had no effect whatsoever upon the excitability curve for the white test-light applied subsequently.

4. It was confirmed that the capacity to produce retinal induction is greatest in a retinal area 15° from the fovea.

5. Some experiments suggesting decremental propagation of spreading induction were carried out.

The writers wish to express their deep gratitude to Prof. K. Motokawa for many helpful criticisms and suggestions.

References

1) Motokawa, K., J. Neurophysiol., 1949, **12**, 475.
2) Motokawa, K., *ibid.*, 1950, **13**, 413.
3) Motokawa, K., *ibid.*, 1951, **14**, 339.
4) Motokawa, K., J. Exp. Psychol., 1953, **45**, 369.
5) Motokawa, K. & Ebe, M., *ibid.*, 1953, **45**, 378.
6) Motokawa, K., Iwama, K. & Ebe, M., Jap. J. Physiol., 1952, **2**, 198.
7) Motokawa, K., & Ebe, M., Nature, 1952, **170**, 79.
8) Motokawa, K. & Ebe, M., Science, 1952, **116**, 92.
9) Motokawa, K., Iwama, K. & Ebe, M., Tohoku J. Exp. Med., 1954, **59**, 11.
10) Motokawa, K. & Ebe, M., J. Neurophysiol., 1954, **17**, 364.
11) Motokawa, K., *ibid.*, 1949, **12**, 291.
12) Motokawa, K., Nakagawa, D. & Kohata, T., J. Comp. Physiol. psychol. (in press).
13) Kurosawa, T. & Katayama, S., Tohoku J. Exp. Med., 1956, **63**, 137.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

New Facts on Neutralization of Retinal Induction

By

Dairin Nakagawa and **Tadashi Kohata**
(中川大倫) (木幡正)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, December 16, 1955)

Introduction

A physiological effect remaining within a retinal area, after exposure to any colored light, is called "direct induction." Against this, an effect set up around the retinal area pre-illuminated is called "indirect induction."[1] Retinal induction was shown by Motokawa[2] to be a basic physiological process underlying a variety of psychological phenomena such as contrast,[3] optical illusion,[1] apparent movement,[3] etc. It has been established by Motokawa[4] and by Nakagawa and Kohata[5] that the indirect induction caused by any colored light is extinguished when a remote retinal region is exposed to a patch of complementary color. In the present experiment we became aware that the indirect induction can be extinguished on exposure of a remote retinal region to a stimulus patch of the same color under special conditions. The following investigation was undertaken to specify experimental conditions under which indirect induction is extinguished, and to elucidate the mechanism underlying this phenomenon.

Experimental

Method

Method, procedures and precautions are fully described in other papers,[1),2),4),6-7)] so that only a brief résumé will be mentioned here. After a preliminary dark adaptation of about 20 minutes the electrical sensitivity of the eye was measured at varying moments after the end of pre-illumination. The electrical sensitivity was expressed in terms of ζ which is defined as $\zeta = 100\ (E-E_\circ)/E_\circ$, when E and $E_\circ$ are reciprocals of electric thresholds determined with and without pre-illumination.

The retinal induction effect was determined by the difference between the ζ value for white test-light alone and the ζ value for successive stimuli:

any colored light and white test-light, and it was denominated the contrast effect (C. E.).

In the present experiment the spectral light (equal energy) and color-filtered light were used. The intensity of the latter was given in terms of the illumination measured without the filter by a luxmeter on the stimulating surface. The white test-light was always 10 lux in intensity, if not otherwise stated, and its size was about 1 mm. in diameter.

The distance from the stimulating surface to subject's eye was always 30 cm. The eye used for the experiment was the left one and the right eye was shielded.

The details of the arrangements and procedures will be mentioned together with the results.

Results

At first, it was investigated, how the indirect induction established around a retinal area pre-illuminated was affected by subsequent illumination of the same area with lights of various wave-lengths.

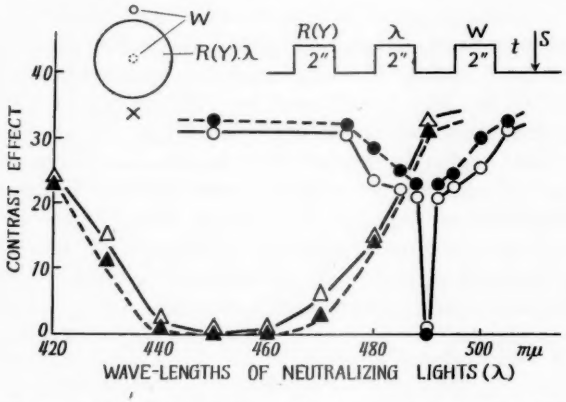


Fig. 1. Neutralization of direct induction caused by red light (R) and by yellow light (Y). Patches used and temporal sequence of stimuli are given in inset, where λ, W, S and t denote spectral tight, white light, electric stimulus, and intervel between W and S respectively. Cross is fixation point. Continuous curves refer to indirect induction, and broken curves to direct induction. Circular and triangular symbols refer to induction caused by red light and by yellow light respectively.

A patch of 12 mm. in diameter was presented on a vertical screen in front of the subjects. The eye was exposed to red (R) or yellow (Y), spectral light (λ) and white test-light (W) in succession, as is shown in the inset of Fig. 1, and then an electric stimulus (S) was applied just at

1.0 (R) or 1.5 (Y) seconds after termination of the white test-light. The time of 1.0 or 1.5 seconds corresponds to the crest time of the ζ-time curve which would be obtained for successive stimuli: red or yellow and white.

The results are shown in Fig. 1. The broken curves connecting solid symbols indicate the neutralizing effect of spectral light upon the direct induction set up by colored light. The detail of this effect is described in our previous paper.[5] The full curves marked by empty symbols refer to the indirect induction around the same retinal area. As can be seen in this figure, the two kinds of curves almost coincide with one another. This fact indicates that the indirect induction around the retinal area pre-illuminated disappears simultaneously when the direct induction within the same retinal area is neutralized.

Then, the question arises as to whether or not the indirect induction established around the retinal image shows a parallel change with the direct induction under other experimental conditions. In the following experiment the direct induction was modified by strong white light applied to the retinal area pre-illuminated by any colored light, and the effect of this procedure upon the indirect induction was measured.

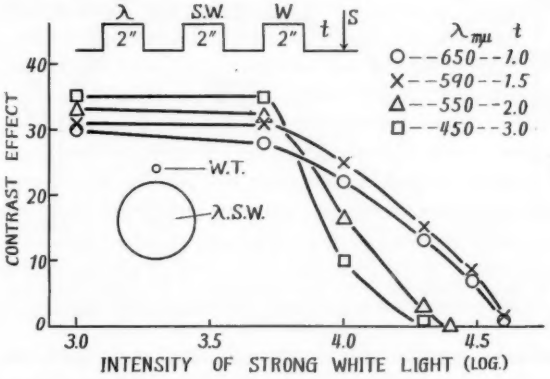


Fig. 2. Effects of strong white light upon indirect induction.
Abscissa: intensity of white light applied prior to white test-light. Intensity of white test-light is 40 lux. In insets S. W. denote strong white light.

The inducing lights used were of wave-lengths 650 mμ, 590 mμ, 550 mμ and 450 mμ. The interval between the white test-light and the electric stimulus was different according to the kind of inducing light, as is shown on top of Fig. 2. In the previous paper[5] we showed that the direct induction is reduced by extremely strong white light applied subse-

quently to the area pre-illuminated with colored light. As is illustrated in Fig. 2, a similar effect can be seen on the indirect induction, although the strong white light was restricted to the retinal area pre-illuminated by colored light. This is another evidence that the disappearance of direct induction is accompanied by that of indirect induction.

In order to see how the indirect induction caused by one patch was affected by another patch presented subsequently at a distant part of the retina, the following experiments were performed. A detector, D, consisting of two parallel bars was used to detect the effect of the spreading induction from the inducing patch, I, presented farther away from D.

The patches used and the order of application of stimuli are shown in the inset of Fig. 3. The color of the detector was red, and that of the inducing patch was varied. The Data obtained are illustrated in Fig. 3.

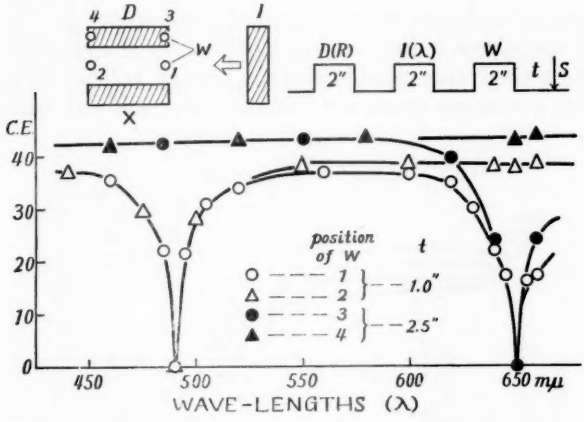


Fig. 3. Relationship between neutralization of direct induction and change of indirect induction as function of wave-lengths of neutralizing spectral lights.

Detector: red horizontal parallel bars. Size of bar is 2×10 mm^2. Distance between parallel bars is 6 mm. Intensity of red light is 20 lux without filter. Inducer: vertical bar of spectral light. Its size is 2×10 mm^2. Distance between detector and inducer is 20 mm. Solid symbols refer to direct induction measured at points 3 and 4. Empty symbols refer to indirect induction measured at points 1 and 2.

When the indirect induction was measured at point 2 between the red bars, neutralization of indirect induction occurred only at 490 mμ (curve marked by empty triangles). This agrees with Motokawa's[4] finding, and based upon the complementary relation between D and I, this kind of neutralization can easily be accounted for. When measured

at point 1, the indirect induction was found to be neutralized not only at 490 mμ, but also at 650 mμ, as is shown by the curve connecting empty circles. In other words, neutralization of indirect induction occurs also with an inducer of the same color as the detector under this special condition. In this case the vanishing of the indirect induction around the detector can not be considered as due to a direct action of the spreading induction from the inducer, because the former and the latter are identical in character.

In the preceding section we observed that the disappearance of indirect induction follows that of direct induction. Then, it is likely that the vanishing of indirect induction at 650 mμ is a secondary effect of the neutralization of the direct induction, because the direct induction and the spreading induction from a red inducer are complementary in character. In order to confirm the neutralization of the direct induction, the measurement of direct induction was carried out at points 3 and 4 with a red inducer, and it was found that the direct induction was missing at point 3, but remaining intact at point 4 (cf. curve connecting solid symbols). The fact that the induction at the left part of the detector remained unaffected is probably due to the circumstance that the spreading induction from the inducer could not reach the point because its energy had been used up for neutralization of direct induction on the way.

This interpretation is supported by the following experiments. The red patches, D and I, used are shown in the inset of Fig. 4. The intensity of the detector was graded in four steps in order to examine the permeating effect of the spreading induction into the field of direct induction caused by the detector. The results are shown in Fig. 4, in which the intensity of direct induction (C. E.) is plotted against the distance from the left end of the detector. All continuous curves indicate that the direct induction decreases as the right end of the detector is approached, and this means that neutralization is easiest at the right end nearest to the inducer and becomes progressively more difficult toward the opposite end. This is because the spreading induction from the inducer loses its energy as it is propagated through the field of direct induction caused by the detector. It is to be noted that neutralization occurs more extensively at the detector of weaker intensity, and this fact may be interpreted in terms of lower resistance against the permeation of the spreading induction. The set of broken curves in the same figure represents the distribution of indirect induction between the two bars of the detector. From these curves it is obvious that the indirect induction changes parallel to the direct induction.

Generally speaking, the indirect induction around the detector can not directly be extinguished with the spreading induction from the inducer of the same color, because the indirect induction is the same character

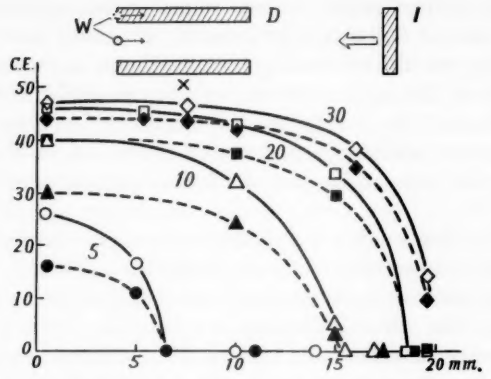


Fig. 4. Neutralization of direct induction and change of indirect induction.

Detector: red horizontal parallel bars. Size of bar is 2×20 mm^2. Distance between parallel bars is 6 mm. Numbers on curves are intensities (in lux) of detector. Inducer: red vertical bar (650 mμ). Its size is 2×10 mm. Abscissa: Positions of measurement indicated by distance from left end of detector. Continous curves refer to direct induction, and broken curves to indirect induction.

as the spreading induction. Nevertheless, the indirect induction can be extinguished indirectly in association with the neutralization of the direct induction. It seems as if the neutralization of direct induction is always connected with the disappearance of indirect induction. However, we have succeeded in separating both phenomena by means of a special device. White parallel bars, BL, were presented in the interval between the detector and the inducer in such a manner that the inner margins of the detector were lined with the white bars (see the inset of Fig. 5 A). The indirect induction remained intact in this arrangement, while the direct induction underwent the same change as mentioned above. (Fig. 5 A). The white bars lining the inner margins of the detector are obviously effective in keeping the indirect induction unaffected which would otherwise be reduced together with the direct induction. The field of direct induction and that of indirect induction are separated by the blocking white bar. It may be supposed that this separation has caused independent changes of the direct and the indirect induction.

In the following experiment the right edges of the bars of the detector were lined with blocking white bars, as is shown in the inset of Fig. 5 B, and similar experiments were caried out. In this experiment both kinds of induction suffered from no change, as is illustrated in Fig. 5 B. The white blocking bars are known to be effective to check the propagation

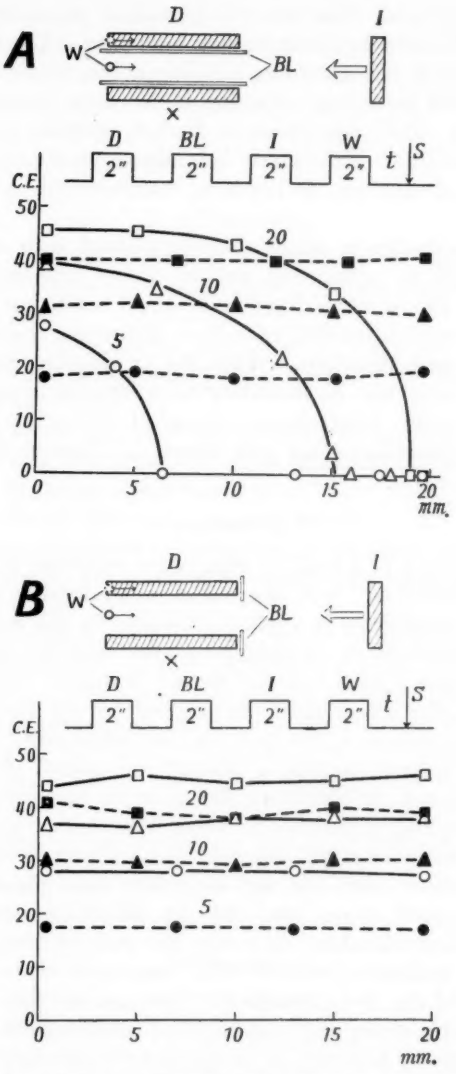


Fig. 5. Experiment on blocking effect of white bar on neutralization of indirect induction

D : red detector, I : red inducer. BL : blocking white bar of 10 lux. W : white test-light. Abscissa : distance from left end of D. Continuous curves refer to direct induction, and broken ones to indirect induction. Numbers on curves indicate intensities of D.

of spreading induction. The direct induction is protected by the white bar from neutralization by the spreading induction. The field of indirect induction is open to the spreading induction, but no neutralization will occur, because the spreading induction is the same character as the indirect induction. This experiment is further evidence that the indirect induction remains intact if the direct induction is unaffected. The reverse is, however, not always true, as has been demonstrated by the experiment in Fig. 5 A.

The latter experiment seems to be explained with such a working hypothesis as will be mentioned below: Some propagating process is initiated at the site of neutralization of direct induction. If this hypothetical process reaches the field of indirect induction, the latter is neutralized (secondary neutralization). When the propagating process is blocked by the blocking white bar, no secondary neutralization of indirect induction occurs, as has really been shown above. It is to be noted that the secondary neutralization occurs only when the detector and the inducer are of the same color.

Discussion

Direct induction is a kind of after-effect surviving a photic stimulus at the site of stimulation. This after-effect is extinguished when the second stimulus of the same color is applied elsewhere in the retina. This phenomenon was ascribed by Motokawa to the neutralizing action of the spreading induction initiated by the second stimulus. The neutralization occurs, because the direct induction caused by the first stimulus is in character complementary to the spreading induction initiated by the second stimulus. The indirect induction around the optical image of the first stimulus is, however, in character the same as the spreading induction caused by the second stimulus. So there is no reason to suppose a phenomenon like neutralization between the indirect induction and the spreading induction. But for the secondary neutralization of indirect induction mentioned above, the indirect induction would survive the first and the second stimulus. In reality the indirect induction disappears with the direct induction because of the secondary neutralization. Thus the after-effect of the first stimulus is wiped out by the second stimulus, unless the first is too strong and long-lasting. The after-effect of a sufficiently strong stimulus is, however, so strong that neither direct induction nor indirect induction caused by it can completely be extinguished by the second stimulus. In such cases a group of psychological phenomena called "figural after-effects" can be seen.[8] In short, the secondary neutralization is a natural device to wipe out the after-effect of a preceding stimulus and to avoid unnecessary interaction and confusion between

the effects of successive stimuli.

As has been shown by Motokawa and Ebe,[3] the secondary neutralization does not take place when the interval between two successive stimuli is so short that apparent movement occurs. The secondary neutralization can be observed only in the stage of succession in which two stimuli are perceived as successive ones.

Summary

Physiological effects remaining within a retinal area and around it after exposure to colored light are designated direct induction and indirect induction respectively.

1. The indirect induction was shown to disappear when the same area was illuminated subsequently with colored light complementary to the pre-illuminating light.

2. The indirect induction was reduced or extinguished also when the direct induction was modified by an illumination with strong white light restricted to the pre-illuminated area.

3. The indirect induction pre-established by the first patch was extinguished when the direct induction was neutralized with the spreading induction from the second patch of the same color presented at a distance subsequently.

4. This fact cannot be accounted for in terms of the neutralization described by Motokawa, because Motokawa's effect could be observed only between two patches complementary to each other. Therefore, it was proposed to distinguish the present phenomenon from Motokawa's and to designate it "secondary neutralization." It was meant by this term that the effect occurs secondarily following the neutralization of direct induction.

5. Some experiments supporting this interpretation were presented.

The authors are greatly indebted to Prof. Motokawa for his kind guidance and for many valuable discussions.

References

1) Motokawa, K., J. Neurophysiol., 1950, **13**, 413.
2) Motokawa, K., *ibid.*, 1949, **12**, 475.
3) Motokawa, K. & Ebe, M., J. Exp. Psychol., 1953, **45**, 378.
4) Motokawa, K., J. Neurophysiol., 1951, **14**, 339.
5) Nakagawa, D. & Kohata, T., Tohoku J. Exp. Med., 1956, **64**, 305.
6) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
7) Motokawa, K., Nakagawa, D. & Kohata, T., J. Comp. Physiol. Psychol., 1956, **49**, 392, 398.
8) Motokawa, K., Nakagawa, D. & Kohata, T., J. Gen. Psychol. (in press).

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

The Effect of Roentgen Rays upon the Electrical Excitability of the Human Eye

By

Juzaburo Umetsu
(梅津 重三郎)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, December 19, 1955)

Introduction

It was reported by Brandes[1] that a sensation of light was evoked by Roentgen rays. Since then this phenomenon has been investigated by Newell and Borley,[2] Belluci,[3] Lipetz[4] and others. Lipetz[4] showed that the Roentgen rays produced a lowering in responsivity of the retina to light and Roentgen rays, taking spike potentials from ganglion cells of the frog retina as an index. Recently Tarusawa *et al.*[5-7] found that the electrical excitability of the eyes decreased following an irradiation of the human body by Roentgen rays for the purpose of Roentgen therapy, and interpreted this phenomenon as a secondary effect of the general serological effect induced by Roentgen ray intoxication. More recently Motokawa *et al.*[8] suggested that such decreases of the electrical excitability of the eyes as observed by Tarusawa *et al.* was a local effect of Roentgen rays on the eyes, instead of a general effect upon the body.

In the present experiment, measurements of the electrical excitability of the eyes after Roentgen irradiation were carried out in order to get more detailed information about the mode of action of Roentgen rays, as revealed by the method of electric flicker.

Experimental

Method

The method of electric flicker was used to measure changes of the electrical excitability of the eyes.

The apparatus used in the present experiment was the same as was described in the previous report. Sinusoidal alternating currents of 20 cps. were applied to the eyes through a pair of silver electrodes of 2×2 cm.^{2} in size, placed on symmetrical parts of the skin of the outer corner of the eyes. The illumination in the whole visual field was 10 lux and was kept

as uniform as possible. The stimulating current was increased automatically from zero at a constant rate of 12 μA per second to determine the threshold for appearance of electric flicker, S_1. The threshold for disappearance of flicker, S_2, was determined by lowering the current from a sufficiently high level at the same rate as above. The difference of both sorts of threshold, S_1-S_2, was denoted by ΔS. The time course of ΔS was studied after Roentgen irradiation of the eyes. For this purpose four or five values of ΔS were measured in succession and their average was plotted as a point in the ΔS-time curve.

Conditions of Roentgen irradiation were as follows: Secondary electric voltage was 70 K.V. Electric current was 4 mA. Filter used was an alminium plate 1.0 mm. in thickness. Area of irradiation 10 × 10 cm.^{2} Distance from the focus to the skin was 30 cm. in the experiment of brief irradiation and 180 cm. in the protracted irradiation, in which a dose of 2.0 r was irradiated for a period of 20 minutes. Dose of scattered Roentgen rays on the eyes was measured by a density meter.

Results

Values of electric flicker, ΔS, were measured before and after a brief irradiation of the eyes with Roentgen rays of 0.5, 1.0 and 2.0 r. The data obtained are shown in Table I. The effect of a Roentgen irradiation

TABLE I

Changes of ΔS before and after Roentgen Irradiation

Dose in r	Subjects	Electric flicker values, ΔS, in μA								
		Minutes after irradiation								
		Before	5	10	15	20	30	40	50	60
0.5	J.U.	15.6	15.0	19.2	22.8	25.2	18.0	18.6	12.9	
	S.K.	13.2	18.0	24.2	24.0		22.2			15.6
	M.K.	18.0	21.6	22.5	24.0	20.4	25.8	19.8		
1.0	J.U.	13.5	21.0	21.0	21.0	24.0	19.8	15.8	10.2	
	S.K.	24.0	26.0	39.6	46.2	34.8	27.0	24.0		
	M.K.	18.6	17.4	21.6	22.8	29.4	19.6	23.4	19.2	
2.0	J.U.	13.2	16.2	23.4	21.0	21.0	24.6	14.4	13.3	
	S.K.	27.6	37.8	36.6	51.6	52.8	49.8	51.0	28.2	27.3
	M.K.	19.8	27.0	30.6	39.6	32.6	33.6	30.6	28.8	24.6

appeared with a latency of some minutes, attained a maximum in about 15–20 minutes and disappeared in about 30–60 minutes. In these cases the time to the crest depended little on the intensity of Roentgen rays,

but the recovery time tended to become longer as the intensity increased. The relation between the intensity of Roentgen rays and ΔS is shown in Fig. 1, in which per cent increases of ΔS above the resting level are plotted against the intensity of Roentgen rays. As can be seen in this figure, the increase of ΔS is almost linear to the increase of intensity of Roentgen rays up to 0.5 r, but the rate of increase declines at the higher intensities. As mentioned in the previous report, the threshold intensity of Roentgen rays to produce a noticeable increase of ΔS was from 0.1 to 0.4 r. The present experiment shows that ΔS depends on the intensity only in a narrow range of intensities, and remains almost independent of the intensity at higher intensity levels.

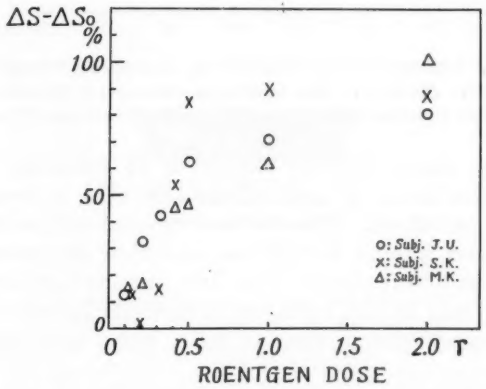


Fig. 1. Effects of intensity of Roentgen rays upon electric flicker values, ΔS. Ordinates: Per cent increases of ΔS above resting level (ΔS_0). ΔS: Difference between threshold for appearance of electric flicker and that for disappearance.

Next, the effect of prolonged exposure upon ΔS was measured, the total Roentgen dose being limited to 2.0 r. The data are shown in Fig. 2, in which arrows A and B indicate the beginning and the end of irradiation. As can be seen in this figure, values of ΔS increased 5 minutes after the onset of irradiation, and attained a maximum in 15–20 minutes to recover to their intial level in 40–60 minutes. Comparing these result with those obtained by brief irradiation of 2.0 r, the latency was much longer in the former case than in the latter, but time courses except the latency were found similar in both cases. Moreover, there was little difference in the increase of ΔS. It seems likely that the effect of Roentgen rays depends on the total dose of Roentgen rays irrespective of stimulus duration.

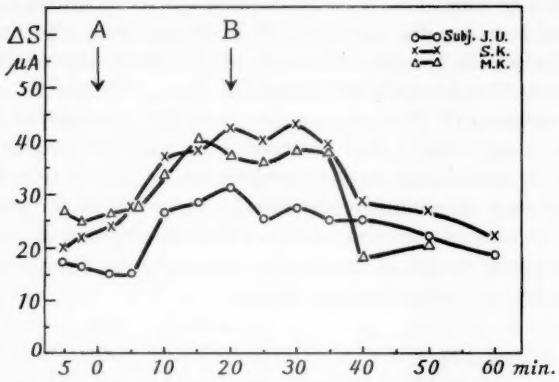


Fig. 2. Changes of electric flicker values, ΔS, following Roentgen irradiation of 2.0 r. Abscissae: Time in minutes from onset of Roentgen irradiation. Arrow A indicates onset of irradiation and arrow B end of irradiation.

In order to assure that the change of ΔS following irradiation of Roentgen rays was due to the local effect on the eyes, a control experiment was carried out as follows: The eyes were covered with a lead plate 10× 10 cm.² in size and 2.5 mm. in thickness, and then ΔS was measured after irradiation as mentioned above. The data obtained are shown in Table II. As can be seen in this table, no remarkable increase of ΔS can be observed after irradiation in doses of 0.5 r, but a noticeable increase of

TABLE II

Changes of ΔS before and after Roentgen Irradiation, Measured with Both Eyes Covered with Lead Plate

Dose in r	Subject	Electric flicker values, ΔS, in μA				
		Minutes after irradiation				
		Before	5	10	15	20
0.5	J.U.	12.0	12.6	13.2	16.8	12.0
	S.K.	19.2	21.0	21.0	17.4	16.5
	M.K.	23.1	19.8	20.4	22.8	16.8
1.0	J.U.	12.0	18.6	18.0	15.0	16.5
	S.K.	20.4	21.0	26.4	24.3	20.1
	M.K.	15.6	22.2	28.5	21.6	13.8
5.0	J.U.	12.0	17.7	21.0	15.0	12.0
	S.K.	13.5	18.0	18.0	17.4	13.5
	M.K.	11.1	16.8	21.0	22.5	17.1

ΔS can be seen after irradiation in doses of 1.0 and 5.0 r. In an attempt to interpret this finding, the following experiments were performed. As can be seen in Fig. 3 A, both eyes were covered with the same lead plate

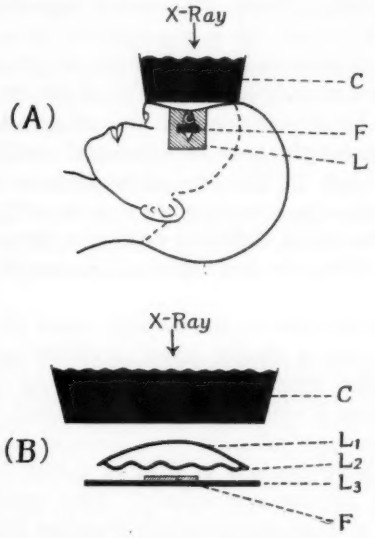


Fig. 3. Arrangement for measuring scattered Roentgen rays. (A) C: Cylinder cone of Roentgen ray apparatus. F: Roentgen ray film. L: Lead plate 10×10 cm^2. in size and 2.5 mm. in thickness. A Roentgen film was placed under the lead plate. (B) L_1: Lead plate 2.0 mm. in thickness. L_2: Lead plate 0.5 mm. in thickness. L_3: Lead plate 2.5 mm. in thickness.

as used in the above experiment and a Roentgen film was inserted between the eyes and the lead plate, and then scattered Roentgen doses were measured by the degree of blackening of the film. Results were as follows: No scattered ray was detected following irradiation with 0.5 r, but appreciable blackening occurred after irradiation of 1.0 r and 5.0 r. Judging from the degree of blackening, the observed dose of scattered rays was equivalent to 0.3 r in the irradiation with 1.0 r and to 0.4–0.45 r in that with 5.0 r. In view of our previous experiment showing that the threshold intensity of Roentgen rays to produce a noticeable increase of ΔS ranged from 0.1 to 0.4 r, it is apparent that the observed increase of ΔS in the latter two cases is due to scattered Roentgen rays. The question now arises as to whether the scattered rays into the eyes originate from the uncovered face region. As is shown in Fig. 3 B, a Roentgen film was placed on a lead plate L_3, 2.5 mm. in thickness, and the film was covered with two lead plates L_1 and L_2. The lead plate, L_1, was 2.0 mm.

in thickness and the other, L_2, was 0.5 mm. in thickness and had a waved surface so as to make a gap between the film and the lead plate, L_1. With this arrangement no scattered rays could be detected after irradiations with 0.5, 1.0 and 5.0 r. From this control experiment it may be said that the scattered rays into the eyes originated from the uncovered face region. As reported previously, no increase of ΔS was observed in the cases of irradiation with 0.5, 1.0 and 5.0 r of the legs, the abdomen and the chest regions. In accordance with this observation no scattered rays could be detected under the same experimental conditions as in the previous experiments. Only in the case of irradiation of the chest region, scattered rays into the eyes were detected in doses of 0.15 r following irradiation with 5.0 r. But this is obviously below the threshold for ΔS-increases. There is no doubt about the local effect of Roentgen rays as the cause of ΔS-increases.

Roentgen rays are said to act directly upon the receptor system of the retina and to cause a similar effect to visible light. Therefore, it is an important question whether or not the increase of ΔS after Roentgen irradiation of the eyes is comparable with the effect of visible light. The eyes of the observer were adapted to a constant illumination of 50 lux, and then the eyes were exposed to light of 1,000, 5,000 and 10,000 lux for a period of ten minutes respectively. After removal of the light the time course of ΔS was measured under constant illumination of 50 lux. The data obtained are shown in Fig. 4, in which the ordinates represent increases of ΔS above the resting level, that is, ΔS-ΔS_0, and the abscissae

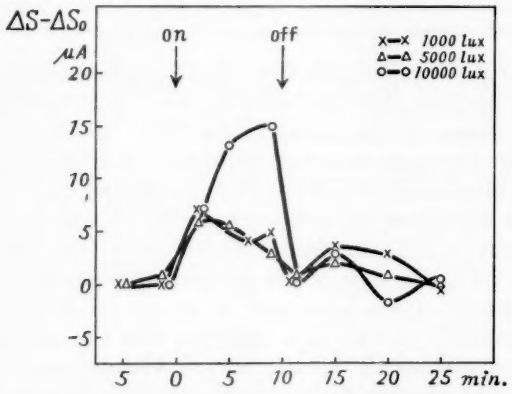


Fig. 4. Changes of electric flicker values, ΔS, following illumination of the eyes with white light. Ordinates: Increases of ΔS above resting level. Abscissae: Time in min. from onset of illumination. Arrows indicate onset and end of illumination.

time in minutes. The data for 1,000, 5,000 and 10,000 lux are marked by crosses, triangles and circles respectively. As can be seen in this figure, values of ΔS increased abruptly after the onset of illuminations of 1,000 and 5,000 lux, but showed a tendency to decrease gradually during illumination. Immediately after removal of the light, ΔS decreased further temporarily, and then increased again slightly to return gradually to the initial level. When the intensity was as high as 10,000 lux, ΔS increased continuously up to the end of the illumination, and after removal of the light very quick recovery resulted. In short, the latency and the time course of recovery were much shorter for visible light than for Roentgen rays. Apart from this difference, it must be emphasized that no sensation of light was caused by Roentgen rays of the dose used in the present experiment, while very strong sensations were caused by ordinary light.

Discussion

As described in the previous report, the threshold intensity of Roentgen rays to produce a noticeable increase of ΔS is lower than the threshold intensity for Roentgen phosphene of the eyes. Therefore, the observed effect of Roentgen rays near the threshold can not be accounted for in terms of the after-effect of visual excitation. It is certain that the increase of ΔS after Roentgen irradiation is due to the direct action of Roentgen rays upon the retina, but not such a general effect as has been assumed by Tarusawa *et al.*, because as mentioned above, the increase of ΔS has a direct connection with the amount of Roentgen rays scattered into the eyes from the other parts of the body. When the dose of scattered rays is over the threshold intensity, the values of ΔS show a noticeable increase after irradiation of the eye covered with a lead plate, but when the scattered rays are below the threshold intensity, the values of ΔS remain unaltered, and the same relation is observed in the experiment for irradiation of other parts of the body. As has been demonstrated by Lipetz, Roentgen rays have a desensitizing action upon the retina to light and to Roentgen rays, and in the present experiment, it has been shown that Roentgen rays also lower the responsivity of the retina to electrical stimulation. This action is caused by such a small amount of Roentgen rays as 0.5 r, and the effect is only temporary.

Summary

The electrical excitability of the eyes following an irradiation of the eyes by Roentgen rays was measured by the method of electric flicker.

1. The effect of Roentgen irradiation appeared with a latency of a few minutes, and attained a maximum in about 15 to 20 minutes to disappear in about 30 to 60 minutes.

2. The effect increased with the intensity of Roentgen rays, but with doses far above the threshold intensity the effect was almost independent of the dose used. The effect depended on the total energy, but little on the stimulus duration.

3. When the eyes covered with lead plates were irradiated by Roentgen rays, some effect could be observed, and it was shown that this effect was due to the direct action of scattered Roentgen rays upon the eyes.

4. The change of ΔS, following illumination with white light for a period of 10 minutes, was measured. In this cases the time course of ΔS was different from that for Roentgen rays. The latency and the recovery time were much shorter than those for Roentgen rays.

I am greatly indebted to Prof. K. Motokawa for his kind guidance and for many valuable discussions.

References

1) Brandes, G., Zbl. Physiol., 1896, **10**, 359.
2) Newell, R. R. & Borley, W. E., Radiology, 1941, **37**, 54.
3) Belluci, B., Radiologia med., 1947, **33**, 141.
4) Lipetz, L. E., Univ. California Radiation Lab. Rep., 1953, No. 2056, 14.
5) Takahashi, K., Abstract of the 10th Jap. Radiol. Congr. (Jap.), 1951.
6) Tarusawa, S. & Takahashi, K., Nippon Hoschasen Gakkai Zasshi. (Jap.), (Nippon Acta Radiol.), 1954, **14**, 423.
7) Tarusawa, S., Takahashi, K., Hosogoe, R., Yanagisawa, T., Sugie, Ch. & Higuchi, K., *ibid.*, 1955, **14**, 784.
8) Motokawa, K., Umetsu, J., Kobayashi, M. & Kameyama, M., Tohoku J. Exp. Med., 1956, **64**, 151.
9) Motokawa, K. & Iwama, K., *ibid.*, 1949, **50**, 319.
10) Motokawa, K. & Suzuki, K., Jap. Med. J., 1948, **1**, 200.
11) Mita, T., Abe, Z. & Byonshik, T., Tohoku J. Exp. Med., 1951, **54**, 45.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Study on Tamura and Takahashi's Disease

(A Hereditary Black Blood Disease)

By

Akira Tamura, Mataro Takahashi, Haruyoshi Ishii
(田 村 彰) (高 橋 又 郎) (石 井 温 義)
and **Seiichi Akashi**
(明 石 精 一)

From the Department of Internal Medicine, Iwate Medical College, Morioka

(Received for publication, January 23, 1956)

This disease which causes the visible mucosae to look dark colored was discovered in a certain village of Iwate Prefecture, and its hereditary study already reported in this Journal.[1] Here we will report our subsequent study of the pathogenesis of this disease.

Experimental

Blood Plasma of the Patients

The cause of the dark appearance of the visible mucosae (e.g., of the oral and the vaginal cavities) in this disease, as has been demonstrated upon obduction, does not lie in any anomaly in the skin, the mucosa or the blood vessels, but in a change of the blood itself. When samples of blood from the patients were separated into plasma and blood corpuscles, the latter showed a very dark red color, while the former was yellow and transparent; and we could foresee that it was free of anomaly. In fact, a spectrochemical examination of the plasma revealed an absorption peak at 280 mμ due to plasma protein, another peak at 415 mμ corresponding to Soret's band due to iron porphyrin and an absorption dome centering at about 460 mμ due to bilirubin, in samples from both normal subjects and patients of this disease. An examination of protein fractions by cataphoresis also showed only the figure of cataphorized albumin and α, β and γ globulins and no fraction of plasma specifically due to this disease. The plasma iron content again showed an approximately normal value.

Blood Corpuscles

When the plasma is removed from the blood of these patients, the difference of the color of the blood corpuscles from the normal is ac-

centuated; and we are led to attribute the disease to an anomaly in the blood corpuscles.

When the stroma is separated out of the corpuscles, it is light yellow and translucent, as in normal blood, but the hemoglobin precipitated in the lower layer is not of the bright red color of normal blood cells but of specific gravy color, so that we could infer that an anomaly is present in the hemoglobin of the patient. Using such hemoglobin, we made the following experiments.

Serological examination of hemoglobin (including blood cell stroma)

We made experiments using rabbits in order to investigate the serological nature of globin in nigremia.

We injected the blood of patients of this disease under the skin of each rabbit, and carried out the precipitation using the serum which was separated from the blood of these rabbits in both the patients of this disease and the normal subjects. But we could make out no difference between the two cases about precipitin titer, precipitin quantity, and absorption test.

Crystals of hemoglobin

Kusunoki's method was used in preparing hemoglobin crystals from the blood samples of normal subjects and of the patients of this disease, but no difference was confirmed.

Electrophoretic picture of hemoglobin of the patient

Using a Tiselius' apparatus a suspension of the hemoglobin of the patient was cataphoretized, but only the main peak A+B of the fast component B and the slow component A was observed, as is normal with hemoglobin.

Paper chromatography

The hemoglobin was hydrolysed by addition of hydrochloric acid, then the acid was removed and the decomposed hemoglobin was dropped on filter paper, which was developed two-dimensionally with phenol and *n*-butanol acetic acid, but only the coloring due to cystine and tyrosine appeared more prominent than in the normal. No heterogenous amino acid specific to the disease was observed by this method.

Absorption chromatography

Samples of blood cells of normal and diseased subjects, of which the stroma was removed, were relieved of their globins by the action of hydrochloric acid and samples of heme liquor were obtained. This liquor was poured into a glass tube filled with calcium carbonate and when adequate suction was applied, as shown in Fig. 1, with normal heme, a gray brown absorption layer and a light brown one under it appeared in the upper part of the calcium column, but with heme of the patients, two absorption layers, a somewhat thicker layer of dark green

and a thin layer of brown appeared instead of the gray brown layer of absorption of normal heme. Thus, we were first led to suspect an anomaly in the heme, but upon isolating these layers with care and examining their absorption spectrum, we found they both caused an absorption curve identical with hematin to appear, showing that they represent layers of aggregated hematin. It is, however, unknown why such abnormal color layers of hematin appear in heme of the patient of this disease. (Fig. 1, 2, 3, 4, 5)

Hemoglobin iron content

The hemoglobin iron content of the patient proved approximately normal. From the above examinations and tests, we have deduced that the anomaly causing this disease lies in the erythrocytes, and in particular, in the hemoglobin of the patients. Further, the globins of their hemoglobin show no anomaly under crystallographic, serological, electrophoretic

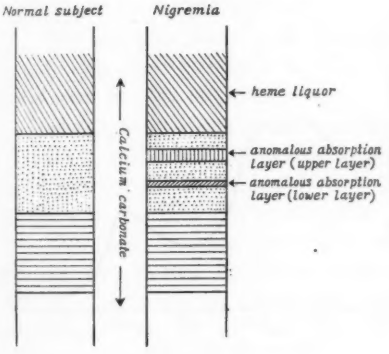


Fig. 1. Absorption chromatography. The heme liquor was poured into a glass tube filled with calcium carbonate used as absorption chemicals, and when adequate suction was applied, with normal heme, a gray brown absorption layer and a light brown one under it appeared in the upper part of the calcium column, but with heme of the patients two absorption layers, a somewhat thicker layer of dark green and a thin layer of brown appeared instead of the gray brown layer of absorption of normal heme.

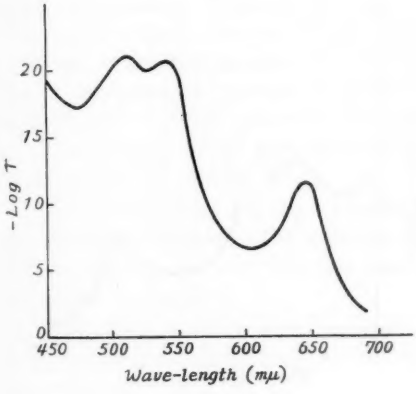


Fig. 2. Absorption spectrum of hematin hydrochloride in normal subjects. Showing from Fig. 2 and Fig. 3 it is found that nigremia and normal subject both caused a absorption curve identical with hematin to appear.

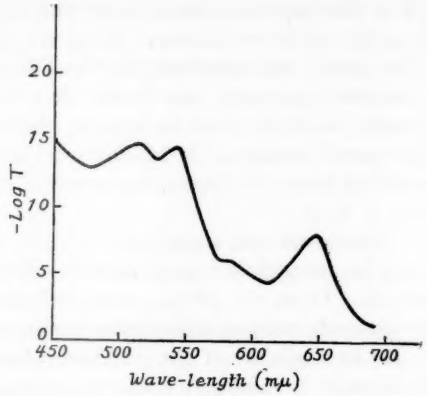


Fig. 3. Absorption spectrum of ehmatin hydrochloride in nigremia.

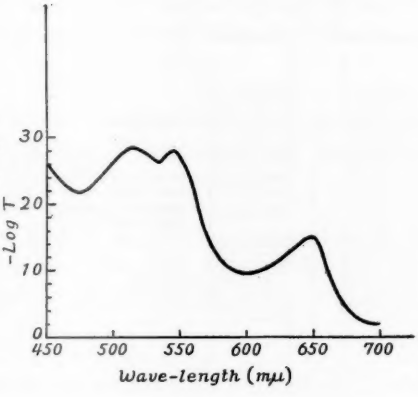


Fig. 4. Absorption spectrum of anomlous absorption layer in nigremia. Upon isolating the upper thicker layer of dark green by aceton hydrochloride and examining their absorption spectrum.

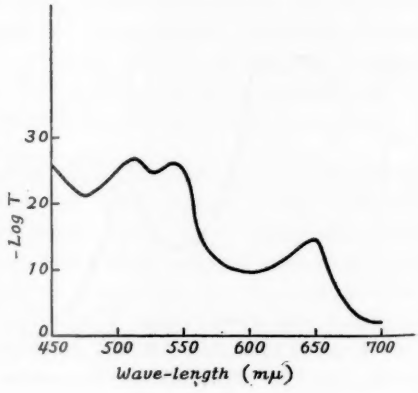


Fig. 5. Absorption spectrum of anomalous absorption layer in nigremia. Upon isolating the lower layer of brown by aceton hydrochloride and examining their absorption spectrum.

and chromatographic tests, but when the globins are removed, the resultant heme shows two anomalous absorption layers not observed with normal heme, which suggests that there is some anomaly in the heme of these patients. Since they are nothing but layers of hematin and since it is unknown what causes these layers to appear, the blood of the patient of this disease seems to be abnormally oxidizable.

Spectrum of Blood of the Patient

Since the blood of the patient appeared much darker than that of normal subjects, it was anticipated that this change in color tone might be traced spectrochemically, and the following experiments were made.

The absorption spectrum of the patient's blood in the ultra-violet region

Blood was sampled from the earlobes of the patient and normal subjects, diluted with distilled water down to 1:2,500 and tested with a Beckmann's spectrophotometer.

The samples from both the patients and the normal subjects showed peaks with their summits at 272 and 282 mμ respectively, indicating no difference in this respect.

Absorption spectrum of oxyhemoglobin

0.1% and 0.5% solutions of oxyhemoglobin obtained from the patient's blood were used to the analysis by Beckmann's spectrophotometer. The samples of normal oxyhemoglobin showed peaks of absorption at 345, 415, 545 and 582 mμ, but with samples from the patient, the peak at 345 mμ was shifted by about 5 mμ to the side of longer wave length of 350 mμ. Though no other abnormal absorption band is detected, upon closer examination, we find that the absorption curves centering on the B band at 545 mμ and the A band at 582 mμ are considerably different from the normal. That is, the bands of 470–530 mμ, 560–750 mμ and 590–660 mμ show supernormally high optical density, the absorption curve between 590 mμ and 660 mμ being particularly conspicuous in its height, seemingly suggesting the presence of hemoglobin sulfide, that shows absorption at 620 mμ when the pH value is 8.8, but this high absorption is just in contrast to the high absorption at 470–530 mμ and it is premature to state that the sulfide exists.

Methemoglobin was also suspected in these cases, the pH value of the solutions was lowered to 6.05 and the change in the absorption around 630 mμ was traced, and we found that the optical density remained high, as shown in Fig. 6.

This heightened density, however, is antipodal to the high absorption in the range of 465–530 mμ, so the existence of methemoglobin may be justly denied. (Fig. 6)

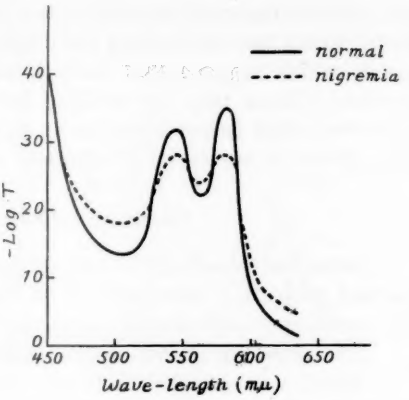


Fig. 6. Absorption spectrum of hemoglobin liquor. The curves show no perceptible difference between the normal and the nigremia.

Reduction of hemoglobin solution

Reducing the oxyhemoglobin in solution by adding hydrosulfite, we found that the oxyhemoglobin of the patients is reduced without residue as that of normal subjects.

Absorption spectrum of methemoglobin solution

When methemoglobin derived by adding potassium ferricyanide to hemoglobin and using phosphoric acid as buffer, keeping the pH value at 6.05, is examined spectrophotometrically, as shown in Fig. 7, the spectrum is different from that of oxyhemoglobin; the peak of absorption at 350 mμ being absent and the maximum peak of the Soret's band at 415 mμ being displaced to 405 mμ. This height of absorption decreases toward the longer wave lengths, making a low peak at 505 mμ and then the absorption curve specific to methemoglobin having the maximum height at 660 mμ begins to appear. The curves of absorption in the range of around 550 mμ to 640 mμ show a perceptible difference between normal samples and those from the patient of this disease.

In normal cases, a dome of absorption with its base at 605 mμ and its summit at 640 mμ appears, but with samples from diseased cases, the absorption curve between 550 mμ and 640 mμ is nearly a straight line, showing no lowering of absorption at 605 mμ as appeared in normal cases.

It is not clarified why such an absorption curve is given, but it may be assumed that it is identical in significance with the supernormally high optical density in the range of 590 mμ to 660 mμ at the foot of the α band on the side of longer wave length. This peculiarity in the absorption curve of hemoglobin of the patients indicates an anomaly in the nature of the hemoglobin in this disease. (Fig. 7)

Absorption spectrum of reduced hemoglobin derived from methemoglobin

As shown in Fig. 8, when the above methemoglobin suspension of

pH 6.05 is reduced by addition of hydrosulfite, the methemoglobin of the patient is completely reduced as in normal cases, showing no anomalous absorption band. (Fig. 8)

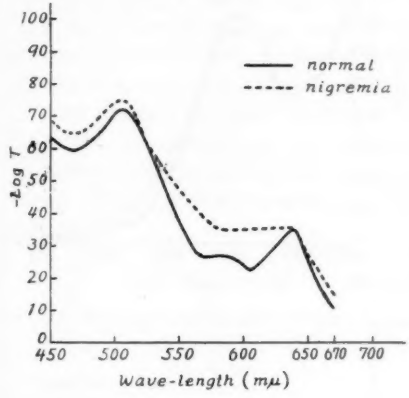


Fig. 7. Absorption spectrum of methemoglobin. 0.1 cc of *M*/100 potassium ferricyanide was added to 0.5% hemoglobin solution diluted with phosphoric acid as buffer, keeping the pH value at 6.05, and accorded absorption spectrum value with 550 mμ. The absorption curve between 550 mμ and 640 mμ with samples from the nigremia is nearly a straight line, showing no lowering of absorption at 605 mμ appearing in the normal.

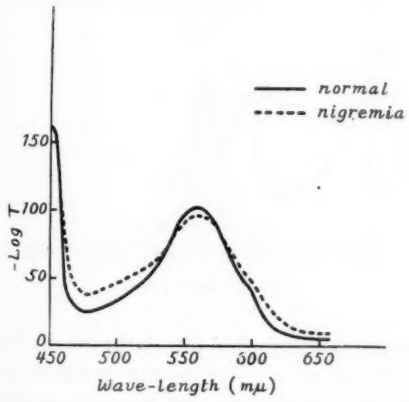


Fig. 8. Absorption spectrum of reduced hemoglobin derived from methemoglobin. When the methemoglobin suspension of pH 6.05 applied in Fig. 7 is reduced by addition of hydrosulfite, the methemoglobin of the patient is completely reduced as in normal cases, showing no anomalous absorption band between the normal and the nigremia.

Absorption spectrum of cyanmethemoglobin

Cyanmethemoglobin was derived from hemoglobin of the patient and examined for any possible abnormal absorption band specific to this disease that might be lurking in the visible part of its spectrum, but no such an anomaly was detected, as shown in Fig. 9. (Fig. 9)

Absorption spectrum of heme

Upon examining the spectrum of heme prepared by removing the globulins from hemoglobin by means of trichlorine acetic acid, the same anomaly which appeared in the spectrum of oxyhemoglobin was observed, as shown in Fig. 10.

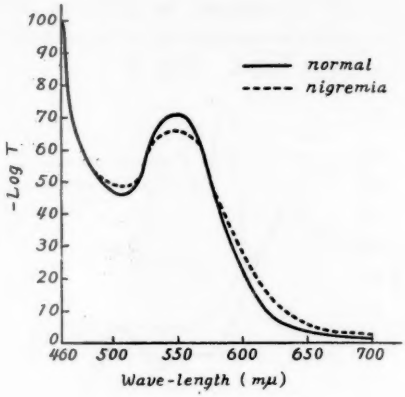


Fig. 9. Absorption spectrum of cyanmethemoglobin. The curves show no perceptible anomalous absorption band in the nigremia to the side of longer wave. But optical density at the foot ranges of both ends of the peak at 545 mμ in the nigremia shows higher than the normal.

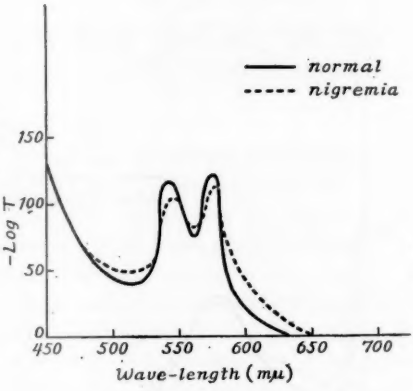


Fig. 10. Absorption spectrum of heme upon examining the spectrum of heme prepared by removing the globulin from hemoglobin by means of trichlorine acetic acid. The darkness of the blood color translates to the heme liquor. This is due to the presence of the variation in the heme itself.

The above spectroscopic examinations show that the darkness of the blood color of the patients of this disease is not due to the presence of anomalous absorption bands specific to this disease and not observable in normal cases, but to the abnormally high optical density at the foot ranges on the both sides of the absorption bands centering on β and α bands of oxyhemoglobin. It was also found that when the pH value is kept at 6.05, the absorption curve of methemoglobin shows a perceptible difference from that of normal cases. (Fig. 10)

Conclusion

The darkness of blood color observed in the patients of this disease was found not to be due to a change in the content of blood iron or catalase, but to an anomaly of the blood corpuscles. Of the corpuscles, however,

the stroma and the globins show no anomaly, and the heme only is assumed to be affected.

Spectrochemical analyses have revealed that anomaly is found in the spectrum of oxyhemoglobin of the patients with its peaks at the β and the α bands, and an anomalous absorption spectrum specific to the disease appears when methemoglobin derived from the patient's hemoglobin is spectroscopically examined.

At present, we have arrived at the tentative conclusion that, inferring from the appearance of two anomalus absorption layers seemingly due to hematin in the absorption chromatograph of heme of the patient, the blood of the patients is more readily oxidized than the blood of normal subjects.

References

1) Tamura & Takahashi, Tohoku J. Exp. Med., 1951, **54**, 209.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

On a New Hematozoic Microbe Resembling Bartonella

By

Akira Tamura, Kazumasa Takahashi, Tokio Sonoda
(田 村 彰) (高 橋 一 正) (園 田 釈 雄)
and **Tsuyoshi Meguro**
(目 黒 強)

From the Departments of Internal Medicine, and of Public Health, Iwate Medical College and Matsuo Mine Hospital

(Received for publication, January 23, 1956)

While examining the urine of a patient of acute nephritis who was hospitalized at the Matsuo Mine Hospital in June 1951, the authors chanced to detect something like a micrococcus vivaciously moving about in its deposit, and under the impression that it had originated in blood, made an examination of the peripheral blood of the patient, which revealed that similar microbes parasitized also in the red blood cells in circulation. Since then, we have made similar examinations whenever nephritic patients have come under our treatment, and have found microbes evidently of the same kind in their urine and blood. Besides, we have found that such microbes are present also in the circulating blood of even apparently healthy denizens of the mining town.

Takahashi,[1] one of the authors of this paper, has previously published a report in the Tokyo Med. J. regarding the examined cases, the bacteriological characteristics of these microbes, and the symptoms of the disease due to them.

We are now engaged in a tireless investigation of the pathogenicity, the endemiologic chracteristics of these microbes and the clinical findings of the disease attributed to them, and we will make an intermediate report of the outline of their characteristics which we have succeeded in elucidating thus far.

Clinical Findings

The findings upon clinical examination of the 43 patients who visited the Matsuo Mine Hospital with the main complaints of edematous or exhausted and enfeebled feeling and were hospitalized with the diagnosis of acute nephritis during the period of June 1951 to May 1954, revealed no perceptible change apparently attributable to these microbes. Excepting that hypertension was not always present, the symptoms were

identical with those of acute glomerular nephritis described in the current text-books of internal medicine. It is also premature, at this stage, to assume that the pathogenic factor lies in the microbes themselves. The following facts, however, presumably stand in causative relation with the microbes.

The frequent presence of urobilinogen in the urine of these cases may be interpreted as being due to the destruction of erythrocytes in circulation by these microbes and absorption of the freed hemoglobin in the liver and the increase of the produced bile pigments, which in turn are excreted as urobilinogen in urine. The transient disappearance of urobilinogen in urine in the course of the disease is perhaps caused by the excess of bile production induced by increased red cell destruction. In respect of the changes of the erythrocytes, we could confirm presence of microcytosis, anisocytosis, and poikilocytosis and hypochromic erythrocytes, and a shift to the left of the diameter of red cells. Though these anomalies cannot be finally attributed to the parasitism of these microbes, it is not to be overlooked as a mere secondary anemia.

Sometimes, we observed a rise in the resistance of erythrocyte, an interesting phenomenon not detectable in common anemia.

Experiments on the Microbe

Morphology

The microbes are found in a number varying from 1 to 15 per erythrocyte usually in the form of micrococcus, although they sometimes take a form more resembling a bacillus.

Diameter: they have a diameter between 0.3 and 0.6 μ in erythrocytes, but a diameter even reaching 1.3 μ outside of erythrocytes and in the plasma.

Their motion is a Brownian one moving in all directions.

The erythrocytes parasitized by the microbes are always deformed, some showing the form of bossed spheres, of mulberries, and some the appearance of being on the verge of collapse.

The size of the infested red cells is also subject to change according to the number and the growth of the microbes, ranging from 3 μ in the smallest to more than 9 μ in the largest. Their color, unstained is light yellow with a tint of green, as normal, in the early stage of infection, but in the later stage where the microbes are seen in active motion, the color of the red cells fades further by the decrease of hemoglobin, so that some become nearly colorless. These microbes move about freely in the erythrocytes and in some cases they are seen to press out the surface membrane of the cell to form protuberances. Even in the same patient, the microbes are sometimes numerous and sometimes not, during the

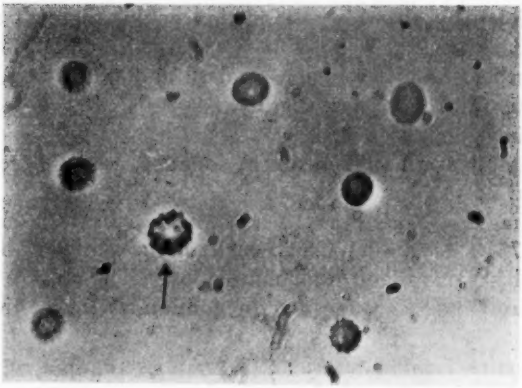


Fig. 1. The erythrocytes parasitized by the microbes are deformed and the microbes are on the middle and verge of them. ×1200

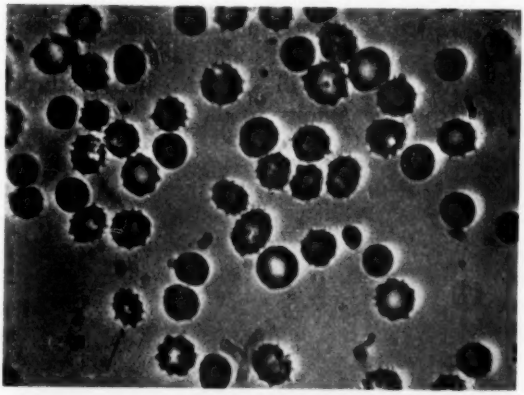


Fig. 2. The erythrocytes parasitized by the microbes. ×1200

course of the disease. Sometimes, they are found wandered out of the erythrocytes into the plasma. (Fig. 1, 2)

Stainability. 1) By common staining method: The microbes stain well by methyleneblue, fuchsin, gentianaviolet and Bismarck-brown.

2) Gram's staining: negative.

3) Blastocyte, flagella and capsule staining: negative by Moller, Toda and Hiss' methods used for the purpose respectively, indicating absence of such structures in the microbes.

4) Romanowsky's staining: Giemsa's and May-Grunwald's solutions were used. The infested erythrocytes stained light red or yellow-greenish red in a tone lighter than the normal, and the microbes violet

or reddish violet, by absorbing the methyleneblue pigment abundantly.

Biochemical assay

The microbes were tested for catalase and peroxidase, and showed positive reactions, though very slightly.

Cultivation

1) On solid and liquid media in common use: The experiment of cultivation failed to bear fruit.

2) In hatched hen's eggs: Fertilized eggs were hatched in an incubator for 7–10 days. 0.1–0.2 cc each of the patients' blood was inoculated in the chorioallantois for cultivation. The culture was renewed every 4–7 days and we succeeded in continuing the cultivation for 24 generations.

The colonies formed on such choroids were opaque hemispheres, humid, greywhite in color, pin-head size in magnitude, somewhat lustrous and homogenous. The choroid under the colonies was incrassated and the blood vessels therein were swollen and serpentine.

As histological findings of the embryonic chickens used in the cultivation, we found the endothelial cells and the histiocytes of the capillary walls injured, and the microbes in the nucleated erythrocytes clustered at the center, as if showing affinity to the nuclei. (Fig. 3, 4)

Fig. 3. Showing a cluster on the chorioallantois in the fourth day after the inoculation in hatched hen's egg.

Animal experiment

Mice of 5–12 g in body weight were used in these experiments, in which whole blood sampled from the cubital veins of the patients were employed for inoculation.

1) Intravenous inoculation: 0.2 cc each of the blood was introduced into the tail vein of the animals. In 3 or 4 days after the inoculation,

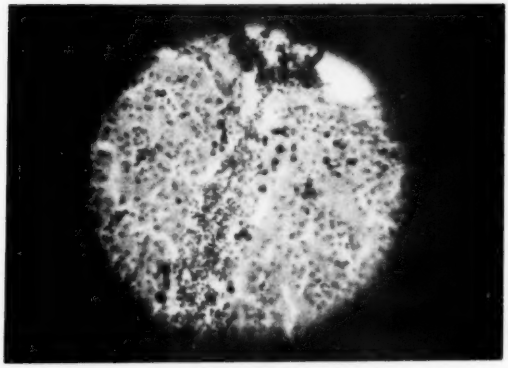


Fig. 4. The liver-tissue of embryonic chicken showing the microbes clustered at the center of the nucleated erythrocytes.

signs of parasitism in their erythrocytes were manifest, and all the 24 mice subjected to the experiment died in around 3 weeks.

Maculae caused by hyperemia and scattered hemorrhages were visible in their intestinal canal.

2) Intra-abdominal inoculation: 1.0 cc each was inoculated. In about a week, the microbes were found infesting erythrocytes in their circulating blood, and all the 11 subjects died in around 3 weeks.

Post mortem examinations showed that the peritoneum was obviously incrassated, adherent and congested. Nothing abnormal was macroscopically observable in their entrails, but in the smear preparations made from sections of their liver and spleen, it was confirmed microscopically that the organs had been evidently invaded by the microbes.

Conclusion

In this report it has been studied upon the bacteriological nature of the subject microbes, but at present time it is unable to classified to the microbes described in the past literature on bacteriology, though we may enumerate Bartonella, Coxiella popiliae and Toxoplasma gondi as proximate types.

Judging from the bacteriological characteristics and the clinical symptoms observed so far, however, we may rule out the two latters and Bartonella bacilliformis, we are not yet in the position to decide either Bartonella muris or Bartonella canis to be responsible as a pathogenic germ.

References

1) Takahashi, Tokyo Med. J. (Jap.), 1955, **72**, 5.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Lateral Summation and Inhibition in the Human Retina

By

Tadashi Aizawa and **Shin'itiro Katayama**
(相 沢 匡) (片 山 新 一 郎)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, January 24, 1956)

Introduction

There is much evidence[1-4] which tends to show that spatial summation takes place in the retina. For instance, Adrian and Matthews[5][6] have demonstrated the interaction of retinal neurones by recording the impulses in the optic nerve of the conger eel. When four points on the retina were illuminated simultaneously, the reaction time of the optic nerve discharge was shorter than when any one of the points was illuminated alone. This effect was similar to the one following an increase in the intensity of the stimulus.

Later, Granit[7][8] determined flicker fusion frequencies for similar four circular test spots together and for each of the spots alone and found spatial summation effects in the human retina. Furthermore, spatial summation in the human retina was studied by Motokawa[9] with increases of electrical excitability by illumination as an index. He has shown that increases of electrical excitability after exposure to five circular spots as indicated in Fig. 1 (A) or to four circular spots, was always higher than those caused by exposure to any one of them alone. The closer the five spots together, the greater the value of increases of electrical excitability. Summation was weaker as the distance separating spots increased, and no indication of interaction was seen at a separation of about 6° in visual angle. Motokawa did not work with greater separations, for he took for granted that no interaction would take place at a greater separation. It was, however, found by chance that the absence of any summation at a separation of about 6° is a phenomenon of transition from summation to inhibition. In the present investigation this relation was established by exploring a much greater extent of retinal areas with two different methods.

Experimental

Methods

Two different methods were used. One is the same as that used in Motokawa's experiment, that is, measurements of electrical excitability of the eye, the other is a binocular brightness match method similar to that used by Schouten & Ornstein.[10]

1) Measurements of electrical excitability of the eye after exposure to light (ζ-method)

The details of the procedure involved are described in Motokawa's[11] paper, so that they may be omitted in this report. Experiments on the human eye were carried out in a dark room after preliminary dark adaptation of about 20 minutes. Following an illumination, the electrical sensitivity of the eye is measured with electrical phosphenes as an index. Percentage increases in electrical sensitivity after exposure to light are denoted by ζ. According to Motokawa[12][13] the ζ-value determined 2 seconds after a white light stimulus lasting 2 seconds represents the magnitude of the cone process, and the ζ-value determined 4.5 seconds after a flash of light represents the magnitude of the rod process.

Such arrangements of test patches as indicated in Fig. 1 (A) and

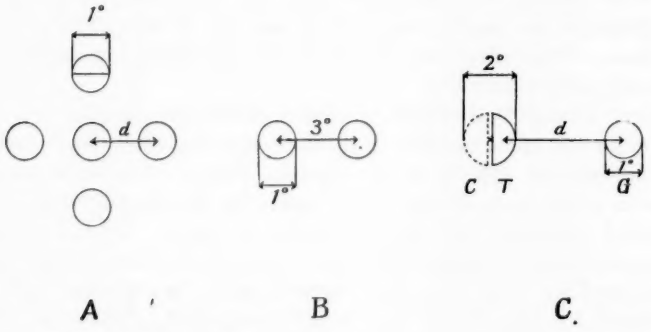


Fig. 1. Arrangement of test patch.

(B) were used in these experiments. Let the value of ζ for the central spot alone be ζ_1, and that for all spots together be ζ_2, then the ratio $\phi = \zeta_2/\zeta_1$ serves as an index of interaction; it indicates summation when $\phi > 1$, and inhibition when $\phi < 1$.

2) Method of binocular brightness match

The aspect of the apparatus used is seen in Fig. 2. Light sources A, B and D are separated from one another with light-tight walls. At distance of 30 cm. from the observer's eye, there are three independent

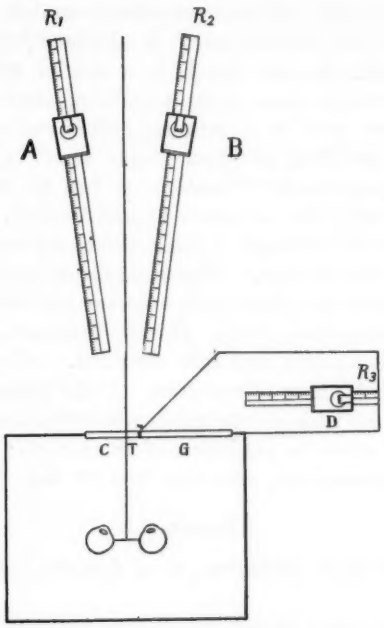


Fig. 2. Apparatus for binocular brightness match method.
A: Light source for comparison patch.
B: Light source for test patch.
C: Comparison patch. D: Light source for glare patch.
G: Glare patch. R_1, R_2 and R_3: Optical benches.
T: Test patch.

patches C, T and G in a perpendicular screen. C and T are semicircular patches of 2° in diameter cut from a sheet of black paper covering the perpendicular screen of ground glass, and placed in such a way that the left eye sees only C and the right one only T. The patch C is illuminated by the light source A of a fixed intensity and serves as a control patch. The test patch T is illuminated by the light source B which is movable along an optical bench R_2. The glare patch G is so arranged that the physical intensity of illumination at the test patch T is not influenced by G. The glare patch G is an opening of 1° cut from a sheet of black paper covering a ground glass screen, which is illuminated with the glare source D movable along R_3. The distance between T and G or the glare angle d can be varied from 2° to 20°.

The experimental room is dimly illuminated, and the eyes of the observer are adapted to this illumination. The experimenter adjusts

the intensity of T roughly till an approximate match between C and T is secured, and then an exacter match is obtained by shifting B slightly forward or backward. In the matching a special difficulty arises with inexperienced observers because of the wandering convergence of the eye axes. This manifests itself in a seemingly different relative position of the two objects in the field of view. After some experience, however, this convergence comes under conscious control so that the two halves of the photometric field can be made to join exactly. Care is taken in the routine measurements always to fixate the exact center of the circular field formed by the two objects. After the objects are matched, the right eye is illuminated with the glare light. So the test field is suddenly seen darker than the comparison field. During exposure, light source B is moved forward until again a match is obtained. All readings are taken 5 seconds after the beginning of exposure. If the intensity of T necessary to match C with and without the glaring illumination be E_2 and E_1, the ratio $\rho = E_1/E_2$ will serve as an index of interaction; the index greater than unity means summation, and that smaller than unity inhibition.

Results

1) Summation and inhibition as a function of distance between stumuli

Such an arrangement of test patches as indicated in Fig. 1 (A) was used in this series of experiment. Each spot had a diameter 1° visual angle, and the distance d between the central and peripheral spots was varied between 1.5° and 11°. The central spot was always centrally fixated. The intensities of illumination were 50, 500 and 5000 lux, and the period of exposure 2 seconds. Electrical sensitivity was determined 2 seconds after the end of illumination, and values of ζ were determined for the central spot alone and for all five together to obtain ϕ. The data are indicated in the upper diagram of Fig. 3 as ϕ-d curves. The ratio ϕ is greater than unity when the distance is small (summation), but decreases with increasing distances to become unity at a certain distance which is smaller for higher intensities. With further increases of d, the ratio becomes less than unity (inhibition). The degree of inhibition is greatest at an intermediate distance ranging 8° to 11°. No indication of interaction could, however, be found at a greater distance such as over 15°.

Similar experiments were carried out in the periphery of the retina, the center of the five spots being placed either at 10° or 20° from the fovea. The intensity of illumination was 500 lux. The results are shown in the lower diagram of Fig. 3. In these peripheral regions of the retina only summation is seen, but no inhibition at all. If the inhibition were to

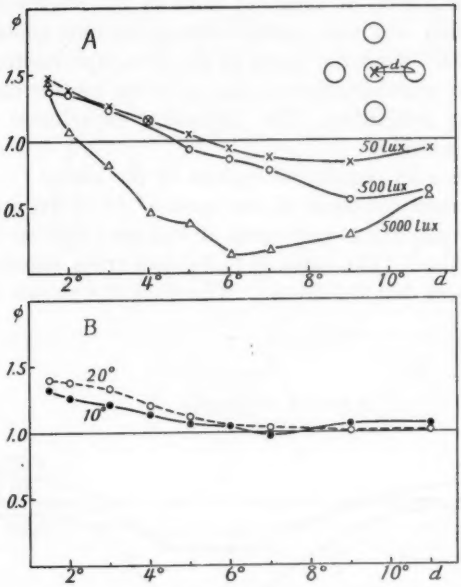


Fig. 3. Relation between index of interaction ϕ and distance between central and peripheral spots d. Upper diagram refers to central fixation of central spot, and lower diagram to peripheral fixation of it. Continuous and broken curves were obtained with central spot at 10° and 20° from fovea respectively. Summation ($\phi > 1$), inhibition ($\phi < 1$). Intensity of illumination was 500 lux.

take place at a certain distance between the central spot and the surrounding spots, it would be expected to take place in the latter experiment as well, because the configuration of the patch used was the same in both experiments. The central spot was situated at the fovea in the first experiment, but at 10° or 20° from the fovea in the second. If the ζ_1-value for the central spot alone were definitely lower for some reasons at the periphery than at the fovea, the ratio ζ_2/ζ_1 would always be greater than unity. The summation observed in the second experiment might be an apparent one due to such a simple reason. In reality, however, the ζ_1-values obtained were 57, 55 and 50 at the fovea, 10° and 20° respectively; the difference is obviously too small to account for the great difference observed between the two experiments. The highest value of ϕ was 1.5 and the lowest 0.54 in the first experiment, whereas the highest and the lowest were 1.4 and 0.98 in the second. Thus, the amplitude of ϕ is far greater in the first experiment than in the second. This difference cannot

be accounted for merely in terms of shifts of the reference values ζ_1.

The inhibition was very conspicuous when four spots fell upon peripheral areas 8°–11° from the fovea in the first experiment. The question now arises as to whether illumination of these parafoveal regions causes especially strong inhibition. The following experiment was performed to answer this question.

2) Inhibition in parafoveal regions of the retina

The patch used consisted of two spots 1° in visual angle with centers 3° apart. This patch was presented at various positions in the temporal horizontal meridian. The ratio of ζ_2 for two spots together to ζ_1 for the left spot alone was denoted by ϕ. The data are shown in Fig. 4 where

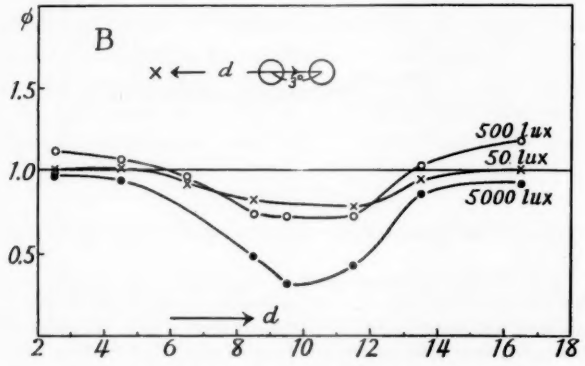


Fig. 4. Regional difference of inhibition determined by two spots method. Abscissae: Retinal position of center of gravity of two spots. Fixation point is marked by cross.

the location of the patch is indicated by the position of the center of gravity of the two spots. A slight degree of summation can be observed near the fovea as well as far in the periphery, but elsewhere inhibition; especially it is strong in the parafoveal region 8°–11° from the fovea.

Similar measurements were carried out in other 7 meridians, the intensity of illumination being fixed at 500 lux. The data obtained are represented by a topogram in Fig. 5. In this figure, the cross hatched area and the dotted area represent a stronger and weaker inhibitory area respectively and the inner and outer blank areas summatory areas.

It should be noted that the stronger inhibitory area covers a zone whose inner and outer diameters 16° and 22°. When two spots fall within this inhibitory area, their effect in terms of ζ is definitely smaller than the effect of either of them. In the five spots experiments we have observed that the effect of the five spots together was much smaller than that of

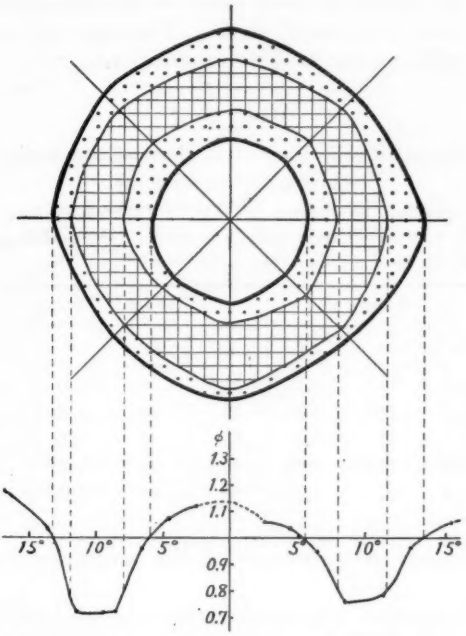


Fig. 5. Topogram of spatial summation and inhibition on the retina. Areas of strong and weak inhibition are cross hatched and dotted respectively. Area of summation is blank.

the central spot alone when the peripheral four spots fell upon the same inhibitory zone. From these facts it is likely that some inhibitory action is initiated within this inhibitory zone when illuminated, and that excitation either within the area or outside the area must be depressed by the inhibitory action thus initiated.

So far the values of ζ was measured 2 seconds after presentation of white light for a period of 2 seconds. This ζ-value represents the magnitude of the cone process. According to Motokawa,[12][13] ζ-values determined 4.5 seconds from the onset of an illumination represent the magnitude of the rod process.

In the following experiment summation and inhibition of the rod process were studied with arrangement of test patches shown in Fig. 1 (B). The pre-illumination was made with a flash of white light lasting 40 msec. The results are reproduced in Table I. All values of ϕ are greater than unity. In other words, there is no indication of inhibition at all, even at such a high intensity of illumination as 300 lux. It is to

be noted that no inhibition takes place of the rod process even within the inhibitory zone mentioned above. Therefore we may mention of inhibition only with regard to the cone process.

TABLE I

Summation in Various Regions of the Retina
(two spots experiment)

Distance between two spots: 3° (Fig. 1 (B)). Preliminary illumination is a flash of white light of 40 msec. ζ_1: ζ for left spot alone. ζ_2: ζ for two spots together. $\phi=\zeta_2/\zeta_1$

Location (degrees from center)	1 lux			30 lux			300 lux		
	ζ_1	ζ_2	ϕ	ζ_1	ζ_2	ϕ	ζ_1	ζ_2	ϕ
5	12.5	14.5	1.12	21.5	38	1.77	19	25	1.32
7.5	14.5	18.5	1.28	20	36	1.80	23	29	1.26
10	15.5	22	1.41	25	43	1.72	23	28	1.21
13	15.5	19.5	1.26	26.5	43	1.62	22	28	1.32
17	19.5	23	1.18	30	43	1.43	23	31	1.34

3) Quantitative measurements of the sensitivity level during exposure

Method of binocular brightness match was used in this series of experiment. Since the experimental apparatus and procedure are already stated in an item of the experimental methods, there seems to be no need to elaborate it again. The illuminations of the test patch T before and during exposure to glare light G are denoted by E_1 and E_2 respectively and the ratio E_1/E_2 is expressed by ρ. The ratio ρ was measured as a function of the intensity of the glare light and the glare angle d. Fig. 6 represents a series of such measurements: Each point gives the mean value of ten readings. The intensity of the glare light was varied between 1.3 and 1000 lux by moving light source D along R_3. It is seen that the glare effect (a decrease of ρ below 1) decreases rapidly with increasing glare angles, and increases as the intensity of illumination rises. It should, however, be noted that there is an inflection on each ρ-glare angle curve except the one for the lowest intensity (1.3 lux). This inflection is seen over 7°–11° glare angles, a retinal region to be identified with the inhibitory zone mentioned above.

In the following experiment the glare angle was fixed at 3°, and the point of fixation was moved along the vertical meridian so as to make brightness matches at various regions of the retina. At each retinal region hundred readings were taken. The data are represented as a function of vertical distances from the fovea in Fig. 7. The intensity of

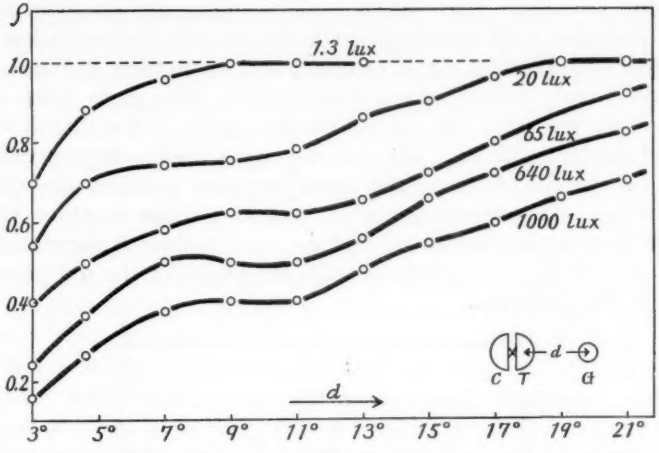


Fig. 6. Glare effect as function of glare angles (brightness match method). Summation ($\rho>1$), inhibition ($\rho<1$).

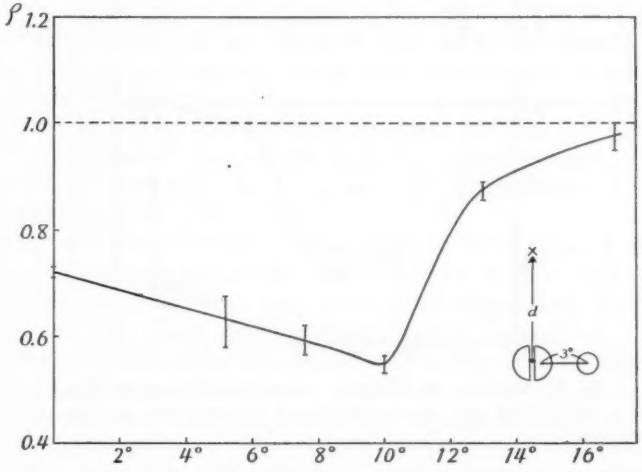


Fig. 7. Regional difference of glare effect.

the glare light was always 1000 lux. The vertical lines on the curve indicate the standard deviation of measurements at each point. The glare effect is strongest in the retinal region 8°–10° from the center in agreement with the result obtained with the ζ-method.

Finaly, two supplementary experiments were carried out so as to

investigate separately glare effects upon scotopic and photopic systems. In one experiment all conditions favorable for selective excitation of the rod system were adopted, e.g. 1. complete dark adaptation, 2. blue working light and 3. use of light of low illumination. The glare patch G was presented on the same side as the control patch C, the illumination of these patches being 0.2 lux. The data are represented in the upper diagram of Fig. 8. Vertical lines on the curve represent standard deviations of 100 readings. In presentation of C and G patches together C looks brighter than in presentation of C alone, that is, there is summation, but not at all inhibition in accordance with the result for the rod process obtained by the ζ-method.

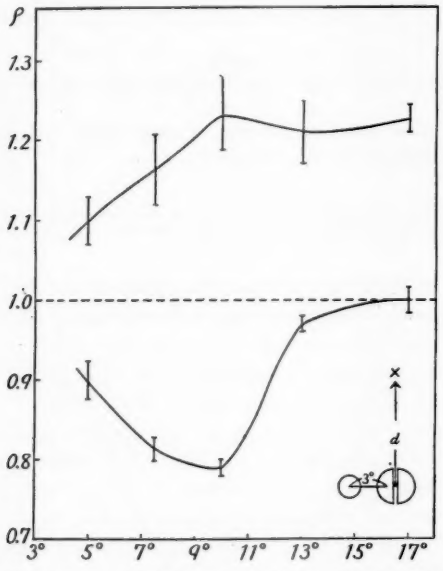


Fig. 8. Variation of brightness caused by simultaneous illumination of a neighboring area in scotopic (upper diagram) and photopic (lower diagram).

Similar measurements were performed with red light of higher intensity, namely 20 lux to excite selectively the cone system. The data are shown in the lower diagram of Fig. 8, which shows a definite inhibition over a parafoveal region in contrast to the data obtained above for the rod system.

Discussion

Kuffler[14)15)] has demonstrated the interaction of different regions

within a single receptive field by simultaneous excitation with two small beams of light. In his papers it was shown that the receptive field was 1–4 mm. in diameter and that functionally the center and the surrounding regions of the receptive field are opposed, the one tending to suppress the other. Therefore it is possible that the inhibition caused by illumination of the parafoveal inhibitory zone in our experiments is related to the peripheral inhibition within a receptive field. However, when the peripheral retina (10° and 20° from the fovea) was illuminated with the five circular spots, no inhibition could be seen at all, as is shown in the lower diagram of Fig. 3. The concept of the inhibition within a single receptive field cannot account for this finding, because there is no reason to suppose that there is no receptive field in the peripheral retina involved.

It is a well known psychological fact that color contrast can be perceived more easily in the parafoveal region than in the fovea. Recently, Kurosawa and Katayama[16] confirmed by the ζ-method that the contrast effect was greatest at 10° from the fovea. They considered that the phenomenon of retinal induction would be based on lateral neural connections in the retina and that the lateral connections must maximally be developed in the parafoveal regions of the retina. The strongest inhibition observed above in the parafovea may be another functional expression of the supposed maximal lateral connections in this retinal region.

Previously, Schouten and Ornstein[10] carried out the same experiment as that illustrated in Fig. 6 by the same binocular method, and concluded that for the glare effect observed by them the neural interaction of the illuminated areas with glare light and test light is more important than the stray light from the glare source.

Boynton and his associates[17] measured by a direct physical method, stray light falling on the foveal center as a function of glare angles in the excised human eye. On their stray light—glare angle curve there is no such inflection as has been observed in our ρ-glare angle curves. Therefore it is difficult to consider that stray light in the eye causes such an inflection of the ρ-glare angle curve. Our data are difficult to interpret in purely physical terms such as stray light, because we have no reason to suppose that scattering of light would be greatest in the parafoveal region where the inhibition is found most conspicuous (Figs. 4, 5, 7 and 8). It is also difficult to interpret from stray light that the cone and the rod systems show quite different behaviours (cf. Table I with Fig. 4 and upper and lower diagrams in Fig. 8). Although our grounds are quite different from those by Schouten and Ornstein, we have reached the same conclusion that the neural interaction is one of the most important factors for the glare effect.

Summary

Spatial summation and inhibition were studied with two methods, Motokawa's ζ-method and the binocular brightness match method.

1. The electrical excitability of the eye was measured after exposure to white five spots together and to the central spot of them alone. The excitability was greater for the five spots together (summation), when the distance between the centrally fixated central spot and other four spots was a few degrees in visual angle, but smaller (inhibition) when the distance was 8°–11°.

2. Distribution of summation and inhibition in the retina was mapped by exploring each region with 2 spots 3° apart from one another, and it was found that the central part of about 12° in diameter was a summation area, which was surrounded by an inhibitory zone of 5° in width.

3. The rod process which was determined by the ζ-method, showed spatial summation, but no inhibition at all.

4. The effect of a glare patch G upon the brightness of a test patch T presented to one eye was measured in comparison with a control patch viewed by the other eye, and it was found that the inhibitory effect of G upon T was greatest when G was presented to the inhibitory zone of the retina determined by the ζ-method.

5. Under experimental conditions advantageous for scotopic vision no inhibition could be found by the brightness match method in accordance with the result obtained by the ζ-method.

The author wishes to express his hearty thanks to Prof. K. Motokawa for his kind guidance and encouragement.

References

1) Piper, H., Zsch. f. Psychol., 1903, **32**, 98.
2) Reeves, P., Astrophys. J., 1918, **47**, 141.
3) Piéron, H., C. r. Soc. Biol., 1920, **83**, 753.
4) Beitel, R. J., J. Gen. Psychol., 1934, **10**, 311.
5) Adrian, E. D. & Matthews, R., J. Physiol., 1926, **63**, 378.
6) Adrian, E. D. & Matthews, R., J. Physiol., 1928, **65**, 273.
7) Granit, R., Amer. J. Physiol., 1930, **94**, 41.
8) Granit, R. & Harper, P., Amer. J. Physiol., 1930, **95**, 211.
9) Motokawa, K., Tohoku J. Exp. Med., 1949, **51**, 179.
10) Schouten, J. F. & Ornstein, L. S., J. Opt. Soc. Amer., 1939, **29**, 168.
11) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
12) Motokawa, K., Ebe, M., Arakawa, Y. & Oikawa, T., J. Opt. Soc. Amer., 1951, **41**, 478.
13) Motokawa, K. & Ebe, M., Tohoku J. Exp. Med., 1951, **54**, 215.
14) Kuffler, S. W., J. Neurophysiol., 1953, **16**, 37.
15) Granit, R., Receptors and sensory perception, Yale Univ. Press, London, 1955.
16) Kurosawa, T. & Katayama, S., Tohoku J. Exp. Med., 1956, **63**, 137.
17) Boynton, R. M., Enoch, J. M. & Bush, W. R., J. Opt. Soc. Amer., 1954, **44**, 879.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Latency of Successive Color Induction

By

Shin'itiro Katayama and **Tadashi Aizawa**
(片山 新一郎) (相沢 匡)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, January 24, 1956).

Introduction

Motokawa's[1] direct and indirect retinal inductions are physiological correlates of successive and simultaneous contrasts respectively. The former is found within a retinal area preilluminated, and the latter in the surrounding areas.

The indirect induction, especially the time course of its development was described in detail in the preceding paper.[2] In the present experiment, we investigated the course of development of the direct induction and the relation between the latency of the direct induction and the intensity of inducing colored light.

Experimental

Method

After a preliminary dark adaptation of about 20 minutes, the electrical sensitivity of the eye was measured with a rectangular pulse of 0.1 sec., taking the least perceptible phosphene as an index.

The effect of the preilluminating light on the electrical excitablity was indicated quantitatively by a percentage increase in electrical excitability above the resting level and represented by ζ. As to the procedure for measuring the electrical excitability the previous papers[3-5] should be consulted.

When red, yellow, green and blue lights are used for preillumination, curves of electrical excitability are obtained with a maximum at 1, 1.5, 2 and 3 sec. respectively after removal of each colored light. The curve for purplish light shows two maxima at 1 and 3 sec. When white light is presented after any colored light, the location of the maximum in the excitability curve as determined after termination of the white light coincides with that for the complementary colored light alone. This is the phenomenon of direct induction. When the interval between the pre-

ceding colored light and the succeeding white light lies within certain limits, from about 0.5 to 20 seconds, the direct induction is independent of the interval. No experiment has, however, been carried out with shorter intervals. Therefore the interval was reduced below 0.5 sec. by means of a sector pendulum to investigate its effect upon the direct induction. The pendulum had two rectangular slits separated by a variable distance, so that the slits passed across a beam of white light one after another as the pendulum swung. The preceding slit was covered with a color filter.

A circular patch of ground glass 1° in visual angle was presented in a vertical plane in front of the subject. The distance between the patch and the eye was always 30 cm. The patch was illuminated uniformly from behind with white light of 100 lux measured with a luxmeter. The pendulum was kept at an elevated position by means of an electromagnet.

When released, the pendulum swung in a vertical plane at a distance of 28 cm. in front of the eye of the subject. Thus one and the same retinal area was illuminated first by the colored light, and then by the white light after an interval which is determined by the distance between the two slits. The duration of the inducing and the white lights was 40 msec. The fixation point of the eye was placed a few centimeters below the opening. Other procedures will be described together with the results.

Results

The broken curves connecting solid circles shown in Figs. 1 and 2 refer to white light alone. The curves connecting half solid circles, empty circles, triangles and crosses represent the data obtained with two stimuli, colored and white, delivered at intervals of 0, 40, 80 and 100 msec. respectively. In the upper diagram of Fig. 1, red light was used as the inducing stimulus. In this diagram, when the interval between the two flashes is very short, the curves are somewhat higher than that for the white light alone and have a maximum at 1.5 sec. (see the curves marked by half solid and by empty circles). When the direct induction is fully developed, the maximum of the curve obtained by the red-white stimulus should lie at 2.5 sec. just like the curve for blue-green light complementary to the red light. Therefore these curves having a maximum at 1.5 sec. mean incomplete development of direct induction. The maximum of the curve becomes higher and is shifted towards 2.5 sec. as the interval is lengthened further (see the curves marked by triangles and by crosses). At greater intervals any further shift of the maximum does not occur beyond 2.5 sec. These facts indicate that it takes certain time for complete development of direct induction. Similar measurements were performed using yellow light as the inducing stimulus. The results are illustrated

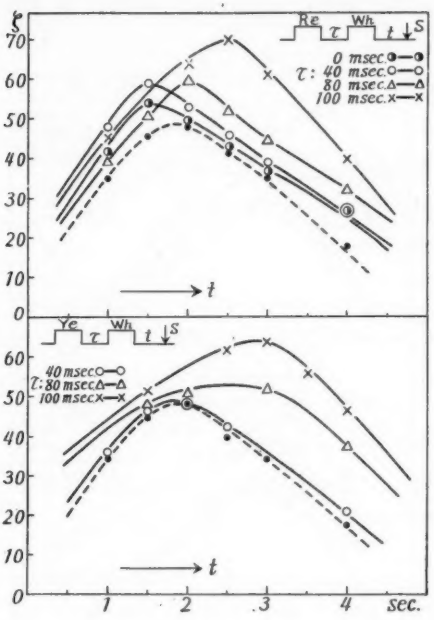


Fig. 1. Direct induction caused by red light (upper diagram) and by yellow light (lower diagram).

Ordinates: percentage increases of electrical excitability above resting level.

Abscissas: time in sec. after end of preillumination. The duration of flashes was 40 msec. Procedure for experimentation is shown in insets where Wh, S and t represent the white light, an electrical stimulus and the interval between Wh and S respectively.

in the lower diagram of Fig. 1. When the interval between the two lights was shortened to 40 msec., the curve obtained almost coincided with the curve for the white light alone (see the curve marked by empty circles). As the interval was increased the maximum of the curve became higher and was shifted towards 3 sec. (see the curves marked by triangles and crosses). 3 sec. are the crest time of the curve for blue light alone.

Next, green light was used as inducing light. The data are shown in the upper diagram of Fig. 2. As can be seen in this figure, the maxima of the curves become more conspicuous at 1 and 3 sec., as the time interval increases. Full development of direct induction can be seen in the curve connecting crosses. A similar curve can be obtained by purplish light alone. The lower diagram of Fig. 2 represents the data obtained with

blue light as inducing light. The final position of the maximum is 1.5 sec.

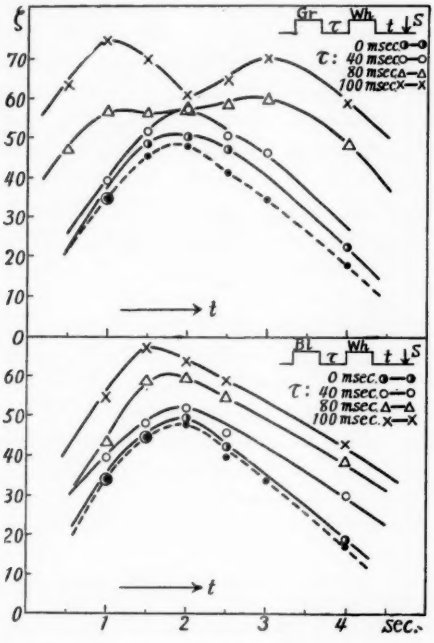


Fig. 2. Direct induction caused by green light (upper diagram) and by blue light (lower diagram).

In Fig. 3, the contrast effects (C. E.) were plotted as ordinates against the time interval as abscissas. The contrast effect is defined as the difference between the maximal ordinate of the excitability curve obtained by the inducing procedure and the corresponding ordinate of the control curve for the white light alone. In these experiments, red light was used as the inducing stimulus. The temporal sequence of the experiment is shown in the inset of this figure. In the upper diagram three curves which were determined with different durations of the conditioning stiumlus are presented. If the latency of development of direct induction is measured from the end of the inducing flash, it is 60, 40 and 40 msec. respectively, when the duration of the red light is 20, 40 and 80 msec. At any rate, the curve increases in height as the interval between the two flashes becomes longer. Furthermore these curves reach a constant level about 130 msec. after the end of the colored light. The following experiment was undertaken in order to investigate the relation between

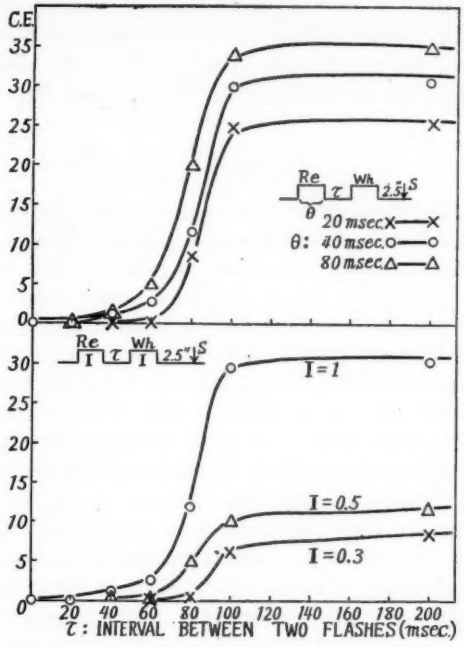


Fig. 3. Dependence of contrast effects on interval between colored and white flashes. τ, θ and I shown in insets represent variable intervals between two flashes, variable durations of inducing flash and variable intensities of two flashes respectively.

the latent period of direct induction and the intensity of light. The results are shown in the lower diagram of Fig. 3. The relative intensities used were 1, 0.5 and 0.3. As can be seen in this figure, the latent period of direct induction is lengthened as the intensity of the two flashes is weaker. Moreover, the weaker the intensity of the two flashes, the lower the curve of direct induction. Some measurements were carried out about the effect of the duration of the white light. The measurements were performed at varying durations ranging from 5 to 40 msec. The intensity of light source was of 100 lux. All the data obtained almost coincided with the curve marked by empty circles in the upper diagram of Fig. 3. This means that the contrast effect is independent of the duration of the white light. Next, the dependence of the contrast effect on the intensity of the white light was studied. The intensity of the white light was varied from 100 to 5 lux. In order to reduce the intensity of the white light, the movable slit was covered by smoked films. The data obtained were

in close agreement with the curve marked by empty circles in the lower diagram of Fig. 3. Thus it was found that the contrast effect is independent of the intensity of the white light as well. From the data stated above, it is apparent that the determinant for the contrast effect is the colored light itself.

Discussion

When the interflash interval was shortened extremely, e.g., to 40 msec. in the experiment with red inducing light followed by white light, the two flashes appeared to the subject as a single flash looking distinctly yellowish. The excitability curve obtained under this condition had a maximum at 1.5 sec. as the curve for the yellow light alone. When the interval between the two flashes was sufficiently lengthened, e.g., to 100 msec., the subsequent white light appeared to the subject as greenish light. The curve obtained under this condition had a maximum at 2.5 sec. like the curve for blue-green light. It seems therefore that the sensation of the subject is faithfully expressed by the shape of the excitability curve.

As is shown in Fig. 3, when the duration of the inducing flash was 20, 40 and 80 msec., the direct induction started 60, 40 and 40 msec. respectively after the end of the inducing light. This finding is not difficult to understand if the off-effect of the light is assumed to be effective for starting direct induction, because the effect is generally weaker for light of shorter duration. There is, however, no evidence that the on-effect is ineffective for starting direct induction. Motokawa's[6] experiment concerning neutralization of retinal induction shows that spreading induction is started at the onset of illumination.

As the direct induction is similar in nature to the spreading induction, both on- and off-effects should be regarded as effective. The latency of direct induction must depend upon summation or interaction of the on- and the off-effects which is a function of the duration of the inducing light.

Summary

The time course of development of direct induction was studied by the method of electrostimulation.

1. Direct induction appears with a latency of about 40 msec., and for its full development it takes about 140 msec. after removal of the inducing light.

2. The latent period of direct induction is lengthened as the intensity and duration of inducing colored light are decreased.

3. The latent period of direct induction is independent of the intensity and the duration of the white light.

Prof. K. Motokawa furnished kind guidance and helpful criticism, for which we express here hearty thanks.

References

1) Motokawa, K., J. Neurophysiol., 1949, **12**, 475.
2) Katayama, S. & Aizawa, T., Tohoku J. Exp. Med., 1956, **46**, 179.
3) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
4) Motokawa, K., Tohoku J. Exp. Med., 1951, **54**, 385.
5) Katayama, S., *ibid.*, 1955, **62**, 209.
6) Motokawa, K., J. Neurophysiol., 1951, **14**, 339.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Physiological Sensation Curves of an Equal Brightness Spectrum

By

Eietsu Suzuki and **Yutaka Ooba**
(鈴木 栄悦) (大場 裕)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, February 1, 1956)

Introduction

Motokawa showed in his previous papers[1-2] that curves very similar to the fundamental sensation curves by König and Dieterici[3] could be obtained from the human eye by measuring the effects of spectral lights upon the electrical sensitivity of the eye determined with electrical phosphenes as an index. Since the curves obtained by Motokawa could be regarded as an expression of real physiological processes in the retina, he termed them "physiological sensation curves." The spectrum used in this experiment was not an equal brightness spectrum, but an equal energy spectrum.

In the present investigation, physiological sensation curves for an equal brightness spectrum were determined with the same method and compared with those for an equal energy spectrum.

Experimental

Method

The electrical supernormality of the eye after exposure to light is taken as a measure for the effect of the light used. The details of the apparatus and the procedure to determine electrical sensitivity are given in Motokawa's paper.[1]

After a preliminary dark adaptation of about 20 minutes, experiments were carried out. The index of excitation was the least perceptible electrical phosphene. Stimulating currents, single constant current pulses of 100 msec. in duration were applied to the eye through a pair of silver electrodes, placed one on the forehead slightly above the eyebrow, and the other on the homolateral temple of the subject. First, the threshold voltage for a dark-adapted eye was measured in such a way that, starting with a voltage sufficient to cause a distinct light sensation, the stimulating

voltage was lowered first in gross steps and then in smaller until finally, the subject could no longer distinguish the stimulus from one far below the threshold. The comparing procedure[4] was used for determination of the threshold. Then the excitability after exposure of 2 seconds to each spectral light was determined in a similar manner. The effect of light was expressed in terms of ζ which was defined by the formula : $\zeta = 100\ (E-E_o)/E_o$, where E and E_o denote electrical excitabilities (reciprocal of thresholds) determined with and without pre-illumination.

An opaque glass disc of 0.6 cm. in diameter was illuminated from behind by spectral light and viewed from a distance of 52 cm., so that the visual angle subtended by the disc at the eye was 40 minutes of arc. The target was always fixated centrally. The spectrum used in this experiment was either of equal energy or equal brightness. The energy was made equal by adjusting the width of the collimator slit of the spectroscope to the prescribed values computed from the known spectral energy distribution of the light source. The brightness was made equal by controlling the intensity of each spectral light with a neutral continuous wedge, so that the CFF-value was the same for all parts of the spectrum.

Results

The luminosity curve (the continuous curve) determined by our method is illustrated in comparison with the photopic luminosity curve determined by Wright[5] (the broken curve) in Fig. 1. The luminosity was adjusted to the level corresponding to a CFF value of 10 cps, where the light-dark ratio of the sector used was 1 :1.

Over the middle range of wavelengths a close agreement is found, although at the longer and shorter wavelength ends of the spectrum our curve is higher than the curve obtained by Wright.[5]

Using the equal brightness spectrum thus obtained, we studied physiological sensation curves with Motokawa's method. The data are shown in Fig. 2 b, in which ζ values determined 1, 2 and 3 sec. after exposure to the spectrum are denoted by R′, G′ and B′ respectively and plotted against the wavelengths of the pre-illuminating spectral lights. For comparison, the physiological sensation curves for an equal energy spectrum obtained from the same subject are illustrated in Fig. 2 a. These data are denoted by R, G and B and found to coincide almost completely with those obtained by Motokawa[1] in his previous paper. For the reason stated in the previous papers,[1][7] the quantities R (R′), G (G′) and B (B′) represent excitation of the red, the green and the blue receptors respectively.

As can be seen in Fig. 2, the curve B′ is highest, the G′ lowest, and the R′ intermediate, that is, the order is different from the sensation curves

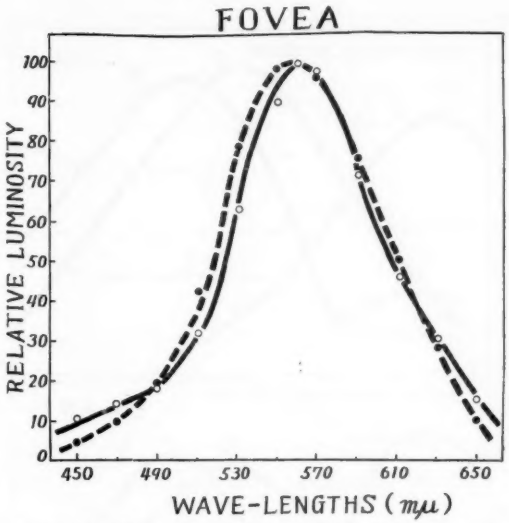


Fig. 1. Luminosity curve obtained at fovea (full line) in comparison with that by Wright (broken line).

for the equal energy spectrum, in which the highest and the lowest ones are G and B respectively. Moreover, the curves of Fig. 2a are much higher than those of Fig. 2b. This is due to the fact that the intensities of the spectral lights had to be sacrificed in order to make the spectrum equally bright.

In determination of ζ values, we encountered often double thresholds in the following manner: As the voltage was decreased, at a certain voltage the subject found it almost impossible to discriminate an electric phosphene from the background of intrinsic light (the first threshold), and on decreasing the voltage further, discrimination became easier, and finally the second threshold was reached. For example when the spectral light of 500 mμ was used for pre-illumination in determining the R- or R′- curve, two kinds of thresholds were obtained. The first threshold (the higher threshold) determined in this way refers to the R- receptor and the second (the lower threshold) to the G- receptor for the reasons stated in the papers by Motokawa[7] and by Motokawa and Isobe.[6] The R- and R′ curves were drawn by the first thresholds. In the following experiments the double thresholds were demonstrated by two different methods.

The spectral lights used were 500, 510, 590 and 650 mμ, and the electric stimulus was delivered at the moment 1 sec. after the pre-illumination of 2 sec. The intensity of phosphene in response to each stimulus

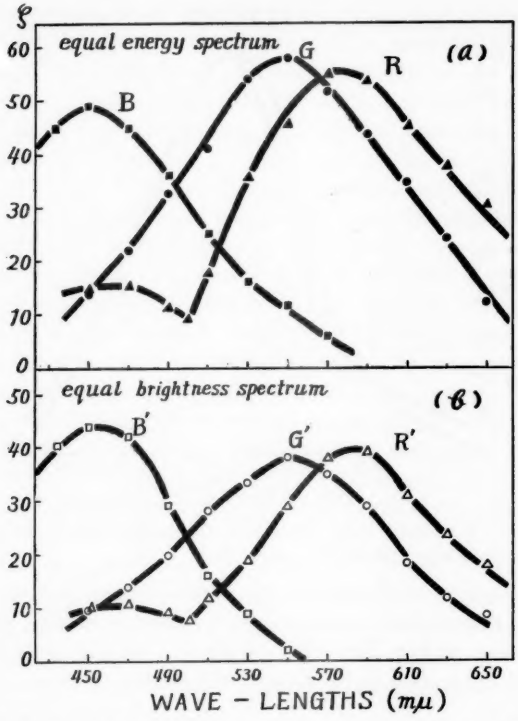


Fig. 2. Physiological sensation curves for an equal-energy spectrum (upper diagram) and those for an equal-brightness spectrum (lower diagram). Ordinates: Percentage increase in electrical excitability of the eye over resting level determined 1, 2 and 3 sec. after end of pre-illumination of 2 sec. Abscissas: Wavelengths of preilluminating spectral lights.

was expressed on a arbitrary scale in such a manner that the intensities of a distinct, an easily noticeable, a faintly noticeable, a just perceptible phosphene (using a comparing procedure), and no phosphene, were denoted by numbers 4, 3, 2, 1 and 0, respectively, and plotted against the stimulating voltage in Fig. 3. Thus, the double thresholds were obtained for spectral lights of 500, 510 and 590 mμ, but for the red light of 650 mμ only a single threshold could be obtained. The thresholds were found at 0.440 and 0.383 volts for the light of 500 mμ, at 0.413 and 0.355 volts for the light of 510 mμ, and at 0.375 and 0.320 volts for that of 590 mμ, while the single threshold for 650 mμ was found at 0.375 volts. The ζ values corresponding to the thresholds stated above are 9.5 and 26 for 500 mμ, 19 and 35 for 510 mμ, 31 and 54 for 590 mμ, and 38 for 650 mμ.

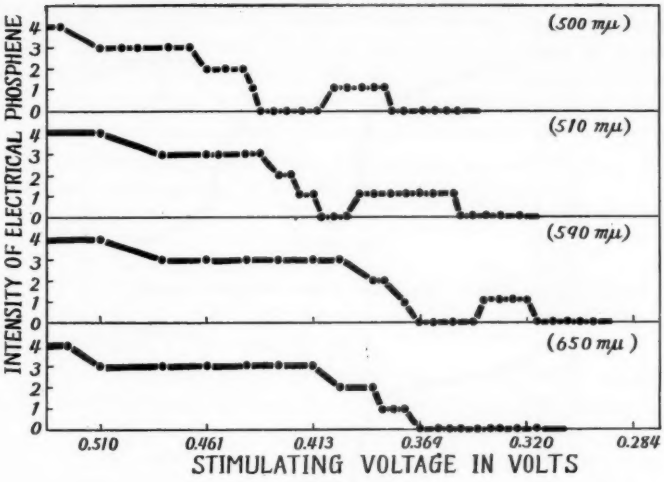


Fig. 3. Intensity of electrical phosphene as a function of stimulating voltages. Spectral lights used were 500, 510, 590 and 650 mμ from top downwards. See text for detail.

We investigated further the thresholds for 590 and 650 mμ with the seeing frequency method, in which the electric stimulus was given twenty times at each voltage to determine the percentage of seeing of phosphenes. Judgment of phosphenes was made with the comparing procedure described by Motokawa.[4] The results are shown in Fig. 4, where seeing frequencies (%) are plotted as ordinates against the stimulating voltages in volts as abscissas. As can be seen in this figure, the value of the seeing frequency is not 100% even at the level of distinct phosphenes. For example, in diagram for 590 mμ the value is 85% above 0.413 volts, and then decreases gradually to 80, 75 and 70% as the stimulating voltage is decreased. It is also to be noted that the value is not zero but about 25–35% even at the lowest level of phosphenes, when the comparing procedure is carried out. The curve rises again at about 0.340 volts, and the seeing frequency ranges between 50 and 65% from this voltage to about 0.320 volts. Then the curve gradually descends as the voltage is decreased. The same is true of the curve for 650 mμ, except that it does not rise again. If the stimulus for 50% of the seeing frequency is taken as a threshold, there are obviously two thresholds for the spectral light of 590 mμ, while there is a single threshold for the light of 650 mμ. We have thus demonstrated the existence of double thresholds by means of the two different methods.

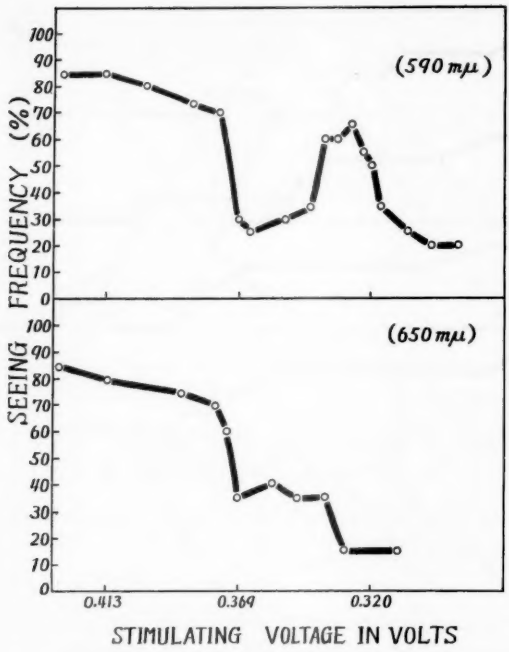


Fig. 4. Relation between seeing frequencies of electrical phosphene and stimulating voltages. Spectral lights used were 590 mμ (upper diagram) and 650 mμ (lower diagram). Experimental procedure is shown in inset.

Discussion

As has been illustrated in Fig. 3, two kinds of thresholds were obtained at the moment 1 sec. after the pre-illumination with spectral lights of 500, 510 and 590 mμ. As the voltage was decreased, discrimination of phosphenes became extremely difficult (the 1st threshold), but easier on further decreases of the stimulating voltage to reach the second threshold. When the second thresholds for the lights of 500 and 510 mμ (the ζ values corresponding to these thresholds are 26 and 35) and the first one for the light of 590 mμ (the ζ value 31) are plotted as ordinates against wavelengths as abscissae, a curve parallel to the G- curve is obtained. This finding indicates that these threshold values used above belong to the G-receptor. Therefore the R- curve was drawn with the first thresholds for the lights of 500 and 510 mμ and the second threshold for that of 590 mμ.

Since the spectral lights of 500 and 510 mμ stimulate more strongly

the G- receptor than the R- receptor, the second or lower threshold is determined by the G- receptor, even when the threshold is measured at the characteristic time interval of the R- receptor, that is, 1 second after the illumination. This is the reason why the R- curve was drawn with the first thresholds in the spectral range from about 490 to 550 mμ. It is without saying that in the other regions the second or true thresholds were used to construct the R- curve.

Summary

The physiological sensation curves for an equal brightness spectrum and an equal energy spectrum were studied by means of the electro-stimulation method at the retinal fovea.

1. The equal brightness spectrum was obtained by adjusting the intensities of spectral lights so as to give the equal CFF value (10 cps.).

2. The physiological sensation curves for the equal brightness spectrum and the equal energy spectrum were compared with each other. The main difference consists in that the " blue " sensation curve is greatest for the equal brightness spectrum, while the " green " sensation curve was greatest for the equal energy spectrum.

3. It was found that two kinds of thresholds were obtained for one wavelength in a certain range of wavelengths, and the question arose which of them should be used for the sensation curves. The mechanism of the double thresholds was studied by the seeing frequency method, and the experimental basis for the construction of the sensation curves was provided.

Prof. K. Motokawa furnished guidance and helpful criticism, for which we express here hearty thanks.

References

1) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
2) Motokawa, K., *ibid.*, 1949, **12**, 465.
3) König, A. & Dieterici, C., Z. Psychol., 1892, **4**, 241.
4) Motokawa, K., Tohoku J. Exp. Med., 1951, **54**, 385.
5) Wright, W. D., Researches on normal and defective colour vision, Henry Kimpton, London, 1946
6) Motokawa, K. & Isobe, K , J. Opt Soc Amer , 1955, **45**, 79
7) Motokawa, K., J. Neurophysiol., 1949, **12**, 475.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Clinical Studies on the Nature of the Auricular Flutter

By

Kazuzo Kato, Mitsumasa Sato, Kenichi Harumi,
(加藤 和三) (佐藤 光正) (春見 建一)
Satoru Murao, Tomohioro Kanazawa, Shigehiko Hanzawa
(村尾 覚) (金沢 知博) (半沢 重彦)
and **Eiichi Kimura**
(木村 栄一)

From the Second Medical Clinic of Prof. Y. Mikamo, University of Tokyo, Medical School, Tokyo and the Medical Clinic of Prof. T. Nakamura, Faculty of Medicine, Tohoku University, Sendai

(Received for publication, February 13, 1956)

It is widely known, that there are two different theories concerning the nature of the auricular flutter; namely the ectopic theory and cirucus movement theory. In the previous papers[1),2)] we attempted to clarify this problem experimentally and reported that the electrically induced auricular flutter is differed from the aconitine-induced flutter in their nature. The analysis of the intrinsic deflections in the direct auricular unipolar leads exhibited to us that in the former the excitation wave propagates in a circus way, while in the latter it starts at the aconitine-injected portion and propagates radially.

Accordingly it was considered that there are two different mechanisms in the experimental flutter which shows the identical figure.

Now it must be examined which of these states corresponds to the clinical flutter. In the literature, the circus movement theory of the auricular flutter was claimed at first by Lewis[3)] in 1921 on the basis of the fact that the electrical axis of the F wave rotates in 360 degrees. Later, Decherd and others[4)] and Grishman and others[5)] also observed the same findings. In recent years, Wenger and others[6)] and Sodi Pallares and others,[7)] analyzing the esophageal, intracavity or precordial leads, obtained the results explanable by the circus theory and claimed the existence of the flutter due to this mechanism.

On the other hand, Prinzmetal and others[8)9)] advocated the ectopic theory on the basis of the nearly synchronous contraction of both auricles in the high speed motion picture recorded during commissurotomy. They pointed out, furthermore, the excitation wave propagates in same direction

both in the esophageal leads and in the direct leads from the auricular surface.

Thus, each of these theories seems to have the firm base. Our purpose in this paper is to solve the problem in the mechanism of the clinical flutter.

Experimental

Material and Methods

Five cases of the auricular flutter administered in the Medical Clinic of Prof. Mikamo, University of Tokyo and in the Medical Clinic of Prof. Nakamura, Tohoku University were analyzed. The clinical findings, the effects of the treatments, etc. are summarized in Table I. The criteria for the diagnosis of the clinical flutter used by us are regularity and uniformity of the F wave with the restless undulation of the baseline. In addition to the usual three limb leads and the six precordial leads, the electrocardiograms were taken from the cavity of the right auricle, the coronary vein and the esophagus at the auricular levels. The former two kinds of leads were carried out by means of the venous catheterization. The modes of the conduction of the auricular excitation were analyzed by comparing the instants of the intrinsic deflections of the F wave in these leads with that of the II Lead recorded simultaneously by multichannel electrocardiograph. The leads from the esophagus and the coronary vein are considered to indicate the excitation of the

Table

Details of the

Case No.	Patient Age	Sex	Duraion of symptoms	Frequency of attacks	Duration of the attack in observation	Aur. rate	Degree of A-V block
1	62	M	8 weeks	One attack	8 weeks	272	2:1
2	41	M	2 years	One attack	2 years	316	2:1 to 3:1
3	62	M	6 months	One attack	6 months	353	2:1
4	22	M	4 or 5 years	Several attacks	3 days	240	1:1 to 2:1
5	49	M	3 months	One attack	3 months	272	3:1

posterior portion of the auricles.

Results of Analysis

Case 1. The electrocardiogram shows the regular F wave of 0.22 sec. in length with the 2:1 auriculo-ventricular conduction, as shown in Fig. 1 and 2.

Analyzing the records, the time references of the intrinsic deflections were arranged in the order from cranial to caudal in the right auricular cavity leads, while from caudal to cranial in leads from the coronary vein and the esophagus, as shown in Fig. 3. The time interval between the intrinsic deflections of the lower and upper portions were estimated as 0.12 sec. in the right auricular cavity leads, 0.10 sec. in the coronary venous leads, and 0.13 sec. in the esophageal leads, respectively. These values correspond to nearly half of the cycle length of the F wave, and the instants of the intrinsic deflections cover the whole length of an auricular cycle. Therefore, it was concluded that the excitation wave in this case propagates in a circus way; from cranial to caudal in the anterior portion and from caudal to cranial in the posterior portion.

Case 2. The electrocardiogram shows the regular F wave, whose length is 0.19 sec. (Fig. 4). The time references of the intrinsic deflections are distributed in the order from cranial to caudal in the right auricular cavity leads and from caudal to cranial in the esophageal leads (Fig. 5).

I

Examined Cases

Etiologic heart disease	Congest. heart failure	Ineffective treatment	Effective treatment	Result of treatment	Remarks
Unknown	+	Carotid sinus or occular pressure, digitalis	Quinidine	Sinus rhythm	
Hypertensive sclerotic heart disease	+	Carotid sinus or occular pressure, Pronestyl, quinidine, digitalis	None	Flutter persists.	Flutter persists over 5 years.
Hypertensive sclerotic heart disease	−	Quinidine, digitalis	None	Flutter persists.	Flutter persists over 3 years.
Unknown	+	Occular pressure	Digitalis, quinidine		Occasional recurrences of flutter attack
Hypertensive sclerotic heart disease	−	Occular pressure	Digitalis	Nodal rhythm to sinus rhythm	Occasional recurrences of flutter

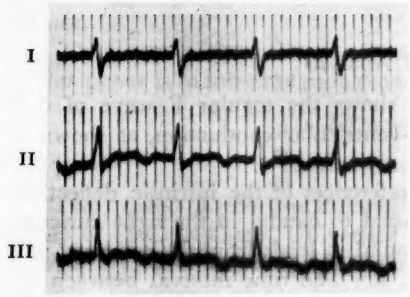


Fig. 1. Case 1. Auricular flutter with 2 : 1 A-V conduction. Auricular rate, 272 per minute; ventricular rate, 136 per minute.

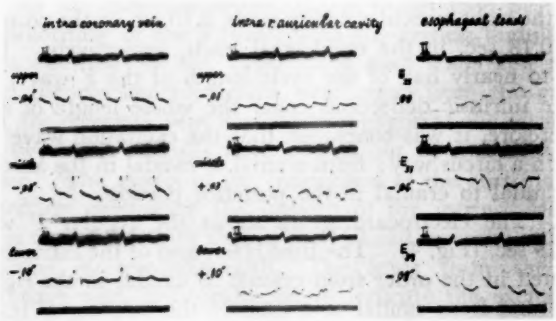


Fig. 2. Case 1. Curves taken from the coronary vein, right auricular cavity and the esophagus, showing the time references of the intrinsicoid deflections in each leads on the left side. The time signal is 0.02 sec.

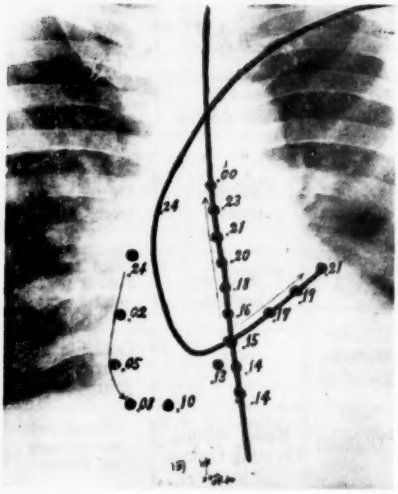


Fig. 3. Case 1. A diagram showing the readings for arrival of the excitation wave. In the right auricular cavity leads the excitation wave propagates from upper to lower and in the esophageal leads and the coronary venous leads from lower to upper. This indicates the circus motion. Auricular cycle length, 0.214 sec.

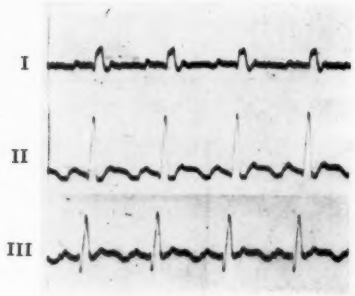


Fig. 4. Case 2. Auricular flutter with 2:1 A-V conduction. Auricular rate, 316 per minute; ventricular rate, 158 per minute.

The time interval between the upper and lower parts were estimated as 0.08 sec. in the esophageal leads and 0.10 sec. in the auricular cavity leads. These values correspond to nearly half of an auricular cycle length. Furthermore, the instants of the intrinsic deflections cover the whole cycle length of an auricular excitation, as shown in the former case. It was concluded, therefore, that the excitation wave propagates in a circus way.

Case 3. The electrocardiogram showed the regular F wave of 0.17 sec. in length (Fig. 6). In the right auricular cavity leads, it was observed that the excitation wave propagtes from lower to upper as shown in Fig. 6 and 7. The instants of their intrinsic deflections are distributed over the interval of 0.10 sec. It corresponds to more than half of the F wave. The esophageal leads failed to explore the auricular excitation in this case. Refering to the experimental findings in our previous paper and the observations in the above mentioned cases, the excitation wave was presumed to propagate also in a circus way.

Case 4. A patient suffering from attacks of arrhythmias and bradycardia of unknown etiology since childhood. The previous electrocardiograms showed sometimes the sinoauricular block and sometimes the nodal rhythm. Recently, an attack of the tachycardia as shown in Fig. 8 occured. This electrocardiogram was at first considered to be the ventricular tachycardia, but after the oppression of the eye-balls, the ventricular rate decreased suddenly and the characteristic F waves became visible in the electrocardiogram, as shown in Fig. 9.

The analysis was carried out by the esophageal and auricular cavity leads recorded simultaneously, and it was observed that the excitation wave propagates from caudal to cranial in the esophageal leads spending 0.12 sec. and from cranial to caudal in the right cavity leads spending 0.13 sec. Therefore the nature of the tachycardia was concluded to be the auricular flutter due to the circus movement also in this case.

Case 5. A patient suffering from mild attacks of angina pectoris.

Fig. 5

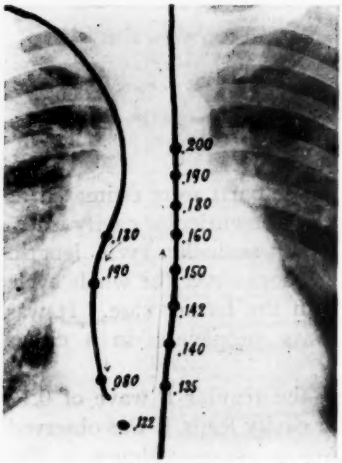


Fig. 6

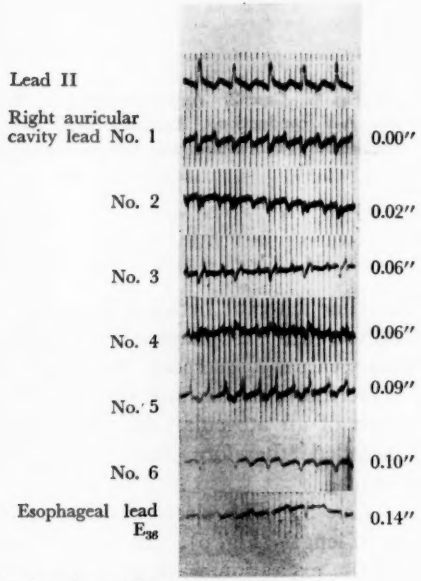


Fig. 5. Case 2. A diagram showing the readings for arrival of the excitation wave, which propagates from cranial to caudal in the right auricular cavity leads, while in the esophageal leads from caudal to cranial. Auricular cycle length, 0.20 sec.

Fig. 6. Case 3. The II, right auricular cavity and esophageal leads. Auricular flutter with 2 :1 A-V conduction. Auricular rate, 353 per minute.

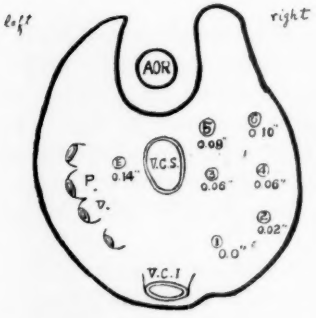


Fig. 7. Case 3. A diagram of the cephalic surface of the auricles, showing the time references of the intrinsic deflections. The excitation wave propagates from lower to upper in the right auricle. The interval between the earliest and the latest intrinsic deflections is 0.10 sec.

In the electrocardiograms the auricular flutter with the regular F wave of 0.22 sec. in length was observed (Fig. 10). Analysis of the esophageal, right auricular cavity and coronary venous leads showed that the excitation wave propagated upwards from lower in every leads (Fig. 11 and 12).

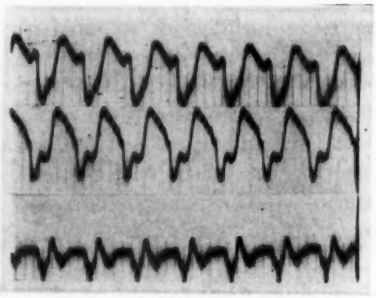


Fig. 8. Case 4, before the occular pressure. Auricular flutter with 1:1 ventricular response. Auricular complexes are indiscernible, and ventricular complexes seem to be the right bundle branch block. Auricular as well as ventricular rate, 240 per minute.

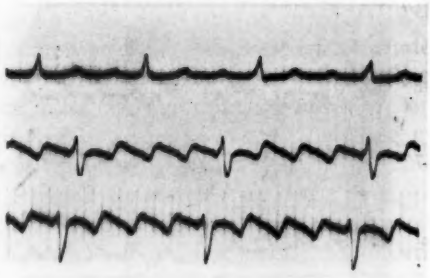


Fig. 9. Case 4, after the occular pressure. Auricular flutter with 3:1 to 4:1 conduction, showing the characteristic saw-tooth appearance of the auricular complexes. Auricular rate, 300 per minute.

The time interval between the earliest and the latest intrinsic deflections was estimated as 0.06 sec. This value is very short in comparison with the values observed in former four cases as well as with auricular cycle length in this case. These findings indicate that the excitation wave starts at the lower protion and propagates radially over the whole auricular muscle within a relatively short period. It is similar to the experimental aconitine-induced flutter.

From these points the auricular tachycardia in this case was concluded as a flutter due to the ectopic rhythm.

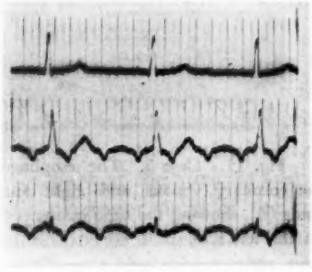


Fig. 10. Case 5. Auricular flutter with 3:1 A-V conduction, showing P′ wave of Prinzmetal. Auricular rate. 272 per minute.

Discussion

The above mentioned analysis exhibits that there are two kinds of

Fig. 11 (A)

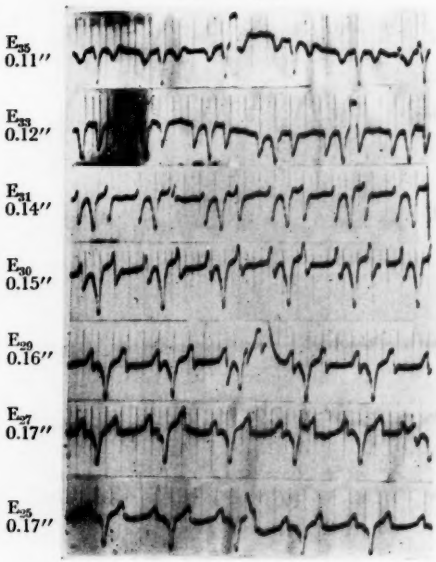


Fig. 11 (B)

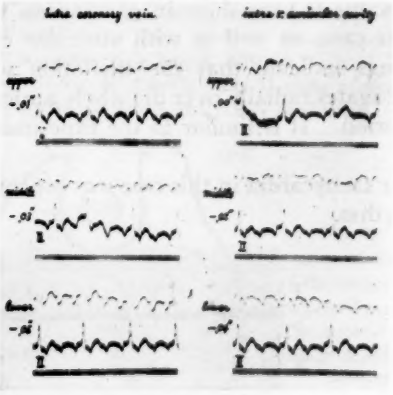


Fig. 11. Case 5. The esophageal leads (A), the coronary venous and right auricular cavity leads (B), showing the time references in each leads on the left side.

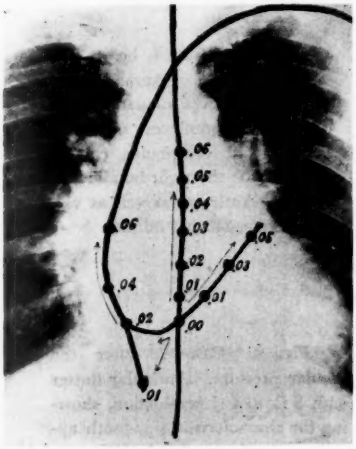


Fig. 12. Case 5. A diagram showing the readings of the intrinsic deflections. The excitation wave propagates from lower to upper in every leads. This indicates the ectopic rhythm arising in the lower portion of the auricles. Auricular cycle length, 0.22 sec.

mechanism in the clinical auricular flutter; in four cases the circus motion of the excitation wave was established and in one case the auricular ectopic rhythm. The findings in the former coincide with those in the experimental auricular flutter induced by the electric stimulation, while those in case 5 with those in aconitine-induced flutter, which were reported in our previous papers.[1)-2)] Among these cases the circus motion in case 3 was presumed by the observation in the right auricular cavity leads alone because of the miscarriage of the esophageal leads, but the distribution of the intrinsic deflections in these leads was similar to those of other cases due to the circus motion.

The differences between these two kinds of mechanism observed in our cases lie in the followings: 1) In the case of the ectopic rhythm the order of the intrinsic deflections are same in various leads and in the case of the circus motion they are reversed in the right auricular cavity lead vs. the esophageal or coronary venous leads. 2) The time interval between the earliest and the latest intrinsic deflections in each lead is shorter in the ectopic rhythm and longer in the circus motion. 3) In the former the intrinsic deflections are distributed within less than half of an auricular cycle length, while in the latter they cover a whole length.

According to Prinzmetal's opinion,[8),9)] if the circus motion exists, the esophageal leads at the mid-auricular level should exhibit two intrinsicoid deflections by the reflections of the two- directional excitatory propagation during an auricular period. Because of the lack of such findings, he denied the circus motion. In our opinion, however, since the esophagus lies rather near the left auricle, the left auricle alone may produce the intrisicoid deflection in this lead and that of the right auricle may not be recognized clearly. Another base of his argument to support the ectopic theory is the fact that the conduction time between the lower and upper portions in the esophageal leads is very short in comparison to the auricular cycle length. Such a finding was observed in our case 5. In other four cases, however, the conduction time is much greater.

The disputation between the circus and ectopic theories, which has been argued for many years, has now arrived to the conclusion that each of the theories are correct and the two different kinds of mechanism present the identical figures in the clinical and electrocardiographic aspects.

The differentiation of the auricular flutter due to the circus motion from that due to the ectopic automatism. It must be necessary to find the signs to differentiate two kinds of the flutter in routine electrocardiograms. Although no differences can be discovered in the clinical findings and the effects of the treatments in our five cases, as shown in Table I, there are some differences in the electrocardiographic findings.

In the first, observing carefully the saw-tooth-like oscillation in the II, III, V_F and V_1 Leads, which are considered as the characteristics of the flutter wave, we might find the downward or upward narrow deflections in them, which correspond to Prinzmetal's P′ wave. Since the V_F Lead may reflect mainly the excitation of the lower part of the auricles and the V_1 Lead mainly the excitation of the middle or upper parts of the right auricle, the longer interval between these deflections in both leads recorded simultaneously suggests the circus motion. Case 1. in which this value is estimated as 0.10 sec., may confirm this relations (Fig. 13). On the other hand, if the instants of these deflections are fairly synchronous as in Case 5 (Fig. 14), the excitation wave is supposed to start at one point

and reach both auricles nearly at the sme time. But these relations are not always definite in all cases.

Secondly, our case 5, which is attributed to the ectopic rhythm, shows the sharp and narrow deflections within the F wave in the II and III Leads. This is not so definite in other four cases.

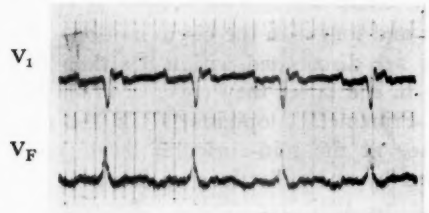


Fig. 13. Case 1. The onset of the intrinsicoid deflection in V_1 precedes the P′ wave in V_F by 0.12 sec.

Fig. 14. Case 5. The onset of the intrinsicoid deflection in V_1 succeeds to the P′ wave in V_F by 0.03 sec.

Summary

1. In five cases of the auricular flutter, the circus motion of the excitation wave was established in four and the auricular ectopic automatism in one.

2. It was concluded that there are two kinds of mechanism in the clinical auricular flutter, as in the experimental observations. This conclusion solves the long lasting disputation between two theories.

3. Some considerations on the differences in the routine electrocardiograms of these two kinds of the flutter were described.

References

1) Kimura, Kato, Murao, Ajisaka, Koyama & Omiya, Tohoku J. Exp. Med., 1954, **60**, 197.
2) Kato, Jap. Circul. J., 1954, **18**, 331.
3) Lewis, Drury & Iliescu, Heart, 1921, **8**, 341.
4) Decherd, Ruskin & Herrmann, Am. Heart J., 1945, **29**, 20.
5) Grishman, Kroop, Jaffe & Steinberg, Am. J. Med., 1950, **8**, 395.

6) Wenger & Hofmann-Credner, Circulation, 1952, **6**, 870.
7) Cabrera & Sodi Pallares, Arch. Inst. Cardiol. Mexico, 1947, **17**, 850.
8) Prinzmetal, Corday, Oblath, Kruger, Brill, Fields, Kennamer, Osborne, Smith, Sellers, Flieg & Finston, Am. J. Med., 1951, **11**, 410.
9) Prinzmetal, Corday, Brill, Oblath & Kruger, The Auricular Arrhythmias, Springfield, C. C. Thomas, 1952.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Effects of the Duration of the Electric Test Pulse upon the Zeta-Value of the Human Retina

By

Takeshi Onoki, Eiichi Onodera
(小野木 健) (小野寺 栄一)
and **Hiroshi Yamamoto**
(山本 弘志)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, February 23, 1956)

Introduction

Motokawa invented[1-2] a new method for analyzing retinal processes concerning chromatic and achromatic vision. The method consists in measuring variations in electrical excitability of the eye after exposure to light. The aspects of variations depend on the wave-length of pre-illumination. When red, yellow, green and blue lights are used for pre-illumination, the eye becomes most sensitive to the electrical test stimulus 1, 1.5, 2 and 3 seconds respectively after termination of the pre-illuminating light. The rod[3-4] or scotopic process has a maximum of its electrical excitability at 4.5 seconds from the onset of the light stimulus. Tukahara and Abe[5] investigated the relation between retinal processes and the stimulating frequency of the repetitive rectangular pulses after a white pre-illumination applied to the dark-adapted eye, and found that the frequencies 20, 36, 42, and 55 cps. were resonance frequencies for the rod process, blue-, green- and red processes respectively.

Motokawa and Ebe[6] studied strength-frequency relation in stimulation experiments of the eye with sinusoidal a. c., and found several minima in the strength-frequency curve. They further showed that the electrical sensitivity to a. c. of 30–37, 40–45, 47–55 and 60–100 cps. was enhanced selectively by blue, green, yellow and red lights respectively. Yonemura and Nango[7] measured the strength-duration curve of the dark-adapted eye and compared it with that of the light-adapted eye. When the illumination was low the ratio of the electric threholds of the light- and the dark-adapted eyes was found maximal at a duration of about 20 msec. From this finding they concluded that the optimal stimulating duration for the rod process would be about 20 msec.

Takahashi, Fujimaki and Yoshino[8] stimulated the eye by sinusoidal

a. c. in a partially illuminated state of the retina. They obtained several minima in the strength-frequency curve similar to those found by Motokawa and Ebe.

In the present experiment we studied the effects of the duration of rectangular pulses which were used as test stimuli in determination of retinal processes.

Experimental

Method

The method consists in measuring the electrical excitability of the eye after a brief exposure to light.

After a preliminary dark adaptation of about 20 minutes, the eye was exposed to a white test patch of 1° in visual angle, then the eye was stimulated with a rectangular single pulse of varying duration. The stimulating current of varying duration ranging from 3 to 200 msec. was obtained by means of a spring rheotome.

The electrical threshold was measured in such a way, that starting with a voltage sufficient to cause a distinct light sensation, the stimulating voltage was lowered in gross steps at first and in smaller steps towards the threshold. Near the threshold comparison procedures were used which consisted in comparison of the effects of the test stimulus and the control stimulus of zero voltage. The index used was the least perceptible light sensation. The difference between the values of electrical excitability (reciprocals of thresholds) with and without pre-illumination was expressed in percentage of the latter, and denoted by ζ. $\zeta=100\ (E-E_o)/E_o$, where E and E_o represent electrical excitability determined with and without pre-illumination. In this way, the ζ values were measured 1, 1.5, 2, or 3 seconds after the end of pre-illumination. ζ values were plotted against the time after termination of pre-illumination. Curves so obtained were called excitability curves.

Results

1) Excitability curves determined by stimulating currents of varying durations

Motokawa[1-2] showed that the shape of the excitability curves obtained by pre-illumination with colored lights were characteristic of the wave-lengths. That is, when red, yellow, green and blue lights were used for pre-illumination, the maxima of the excitability curves lie at 1, 1.5, 2 and 3 seconds respectively. In the present experiment, the central region of the retina was illuminated with white light of 100 lux for a period of 2 seconds, and then the electrical excitability was tested

at 1, 1.5, 2 or 3 seconds with a single pulse of duration ranging from 3 to 200 msec.

In Fig. 1, ζ-time curves or excitability curves determined with pulses of different durations are illustrated. The number of each curve represents the duration of the stimulating current, and the zero-level for each curve is indicated on the left side.

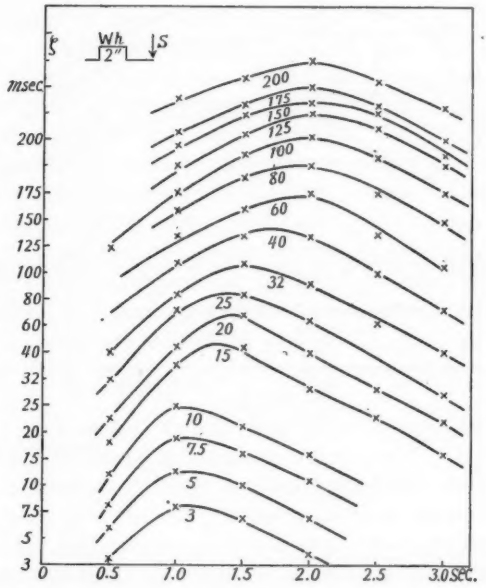


Fig. 1. Excitability curves determined with single test electric pulses of varying durations after the fovea was exposed to light (100 lux). Abszissae: time in seconds after termination of light, Ordinates: ζ values. Number of each curve represents duration of stimulating current, and zero-level for each curve is indicated on left side.

As can be seen in Fig. 1, the curves for durations of 200 to 60 msec. have a crest time of about 2 seconds. As the stimulating duration was decreased, the crest time was shortened by and by from 2 seconds to 1.5 second. The curves for durations of 32–15 msec. have their maxima at 1.5 second. These curves are the same in shape as a curve which would be obtained after yellow pre-illumination. When the stimulating duration is shorter than 10 msec. the curves show their maxima at 1 second, and are in shape similar to a curve which would be obtained by red pre-illumination. It seems that pulses of shorter duration are more favorable for eliciting the red process.

In Fig. 2, ζ-values at 1, 1.5, 2 and 3 seconds of each excitability curves are plotted as ordinates against durations of stimulating cur-

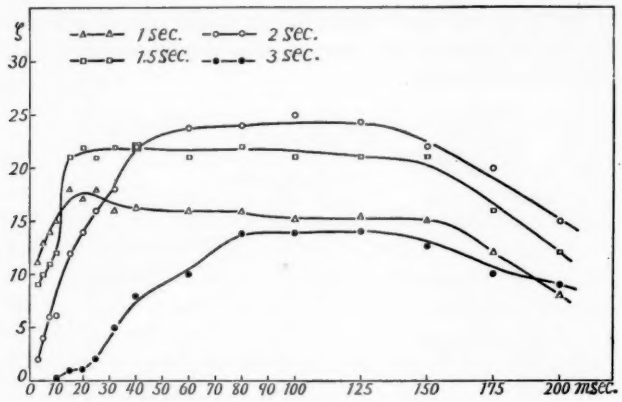


Fig. 2. ζ-duration curves for photopic processes.

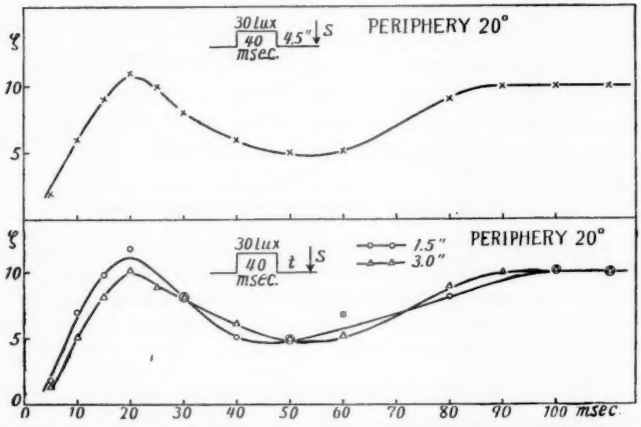


Fig. 3. ζ-duration curves for scotopic processes obtained at 20° from fovea after a white flash of 30 lux and 40 msec. Upper curve: ζ-duration curve for rod process. Lower set of curves: ζ-duration currves for twin process.

rents as abscissae. The curves run parallel when the stimulating durations are long enough. As the duration was shortened, the curve for 3 seconds began to decrease at about 80 msec., and the curve for 2 seconds at 60 msec. The curve for 1.5 second is higher than the others over 32

to 15 msec., but the curve for 1 second is predominant for durations shorter than 10 msec. These results are alike to the relation of ζ values to the stimulating frequency of repetitive stimulation which was studied by Tukahara & Abe. The time constants of color receptor systems must be greater in the order, blue>green>yellow> red, and this assumption conforms not only to our results obtained by single stimulation, but also to Tukahara-Abe's results obtained by repetitive stimulation.

2) Excitablity of the scotopic processes

Motokawa and his co-workers[3-4] showed that the rod process has a crest time of its excitability at 4.5 seconds from the onset of a light stimulus. Oikawa[9] found that scotopic vision is mediated not only by the rod process but also by his so-called twin process. This process consists of a complementary pair of yellow and blue processes and subserves white sensation at low illuminations like the rod process. The crest times of the two components of the twin process are 1.5 and 3 seconds. We used 30 lux white light instead of 100 lux in this experiment. A retinal area at 20° from the fovea was exposed to a white light for a period of 40 msec. and then ζ values at 1.5, 3 and 4.5 seconds were measured.

ζ-duration curves are illustrated in Fig. 3. The upper curve in this figure refers to the rod process and the lower two curves refer to the twin process.

As can be seen in this figure, these 3 curves are very similar to one another not only in shape but also in values of ζ. As the stimulating duration became shorter, ζ values decreased gradually, reached a minimum at 50 to 40 msec., and then increased to make a maximum at about 20 msec. From this fact, it may be said that the stimulating duration of about 20 msec. is most effective for exciting scotopic processes, while those of 50 to 40 msec. are least effective.

In Fig. 4, the ζ values are represented as a function of the intensities of conditioning flashes. Electric pulses of 20 and 100 msec. were used because of their effectiveness for the scotopic processes. As is shown in this figure, the curves for 20 and 100 msec. run parallel. As the light intensity rises by and by from complete darkness, the ζ value increases gradually to reach its maximal value at about 3 lux, remains almost constant till about 50 lux, and then decreases steeply. This finding indicates that the optimal intensity for the scotopic processes is about 3 lux. The depression of ζ values at higher intensities was interpreted by Oikawa as due to inhibition by the cone processes.

Discussion

The previous authors[5-6] showed that the frequency of stimulating currents is important for the study of retinal processes, because it enables

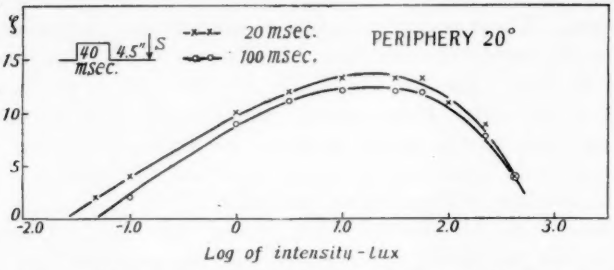


Fig. 4. ζ-log intensity curves for rod process obtained at 20° from fovea with test shocks of 20 and 100 msec. in duration after exposure of 40 msec. flash of white light.

selective stimulation of color receptors. As mentioned above, the red receptor has the smallest time constant and the blue one the greatest. The series, red, yellow, green and blue manifests itself in various phenomena such as crest times, reasonance frequencies, etc. Our findings have added important evidence for the theory of time constants of color receptors. When a pulse of about 10 msec. in duration is used, an excitability curve (Fig. 1) similar to the red curve is obtained. This is because the red receptor is more strongly excited by a pulse of such short duration. With a pulse of 25 msec. a curve similar to the yellow curve is obtained, because the yellow receptor is most sensitive to the pulse of this duration. In this way, selective stimulation of various color receptors would be possible merely by varying the duration of the test pulse. The ζ-duration curves in Fig. 2 not only clarify the ground for the selective stimulation, but also provide the theoretical grounds for Motokawa's method of electrostimulation of the eye, in which single test pulses of 100 msec. are usually used. All curves in Fig. 2 run parallel to one another over a range from 80 to 125 msec. This means that the ζ values are independent of the duration of the test pulse over this range, and that electric stimuli have no such selective action upon the color receptors within this range as in the region of shorter durations. We owe the color-effects revealed by Motokawa's method to the use of an electric test pulse of about 100 msec., which is uniformly effective upon all sorts of color receptors. If a pulse of shorter duration, say, of 10 msec. were used we would always obtain an excitablity curve having a maximum at 1 sec., whatever the color of the pre-illuminating light may be. A pulse of about 20 msec. is most favorable for exciting the rod process. This result is in line with that by Yonemura & Nango.[7] It is, however, to be noted that a pulse of about 100 msec. is nearly as effective as the pulse of 20 msec. Therefore it may be said that in Motokawa's routine method test electric pulses act non-

selectively upon the rod system as well.

Summary

The electrical excitability of the dark-adapted eye was measured by a single pulse of varying durations after exposure to light.

The effect of the light was expressed by ζ, percentage increase of electrical excitability over the resting level.

1. After the eye was illuminated by white light for two seconds, ζ values were maximal 1, 1.5, and 2 seconds respectively after termination of the light stimulus as the duration of test pulse was 3–10, 20–32, and 60–200 msec. In comparison with the data obtained with colored pre-illuminations and a sufficiently long electric test pulse the result was so interpreted that the red, the yellow, and the green receptors were selectively excited by the pulses of 3–10 msec, by those of 20–32 msec. and by those of 60–200 msec. respectively.

2. ζ values measured at 1, 1.5, 2 and 3 seconds after termination of the white light were plotted against the duration of the test pulse, and it was found that ζ values were independent of the duration of the test pulse over a range from 80–125 msec.

It is likely that the selectively excitatory action of an electric pulse upon color receptors is much weaker in the range of duration around 100 msec. than in the range of shorter durations.

3. The dependence of the scotopic processes upon the duration of the test pulse was studied, and it was found that the duration of about 20 msec. was most favorable for the rod process in agreement with the data by Yonemura and Nango.

Prof. K. Motokawa furnished guidance and helpful criticism, for which we express here hearty thanks.

References

1) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
2) Motokawa, K., Tohoku J. Exp. Med., 1949, **51**, 165.
3) Motokawa, K. & Ebe, M., *ibid.*, 1951, **54**, 215.
4) Motokawa, K., Ebe, M., Arakawa, Y. & Oikawa, T., J. Opt. Soc. Amer., 1951, **41**, 478.
5) Tukahara, S., & Abe, Z., Tohoku J. Exp. Med., 1951, **54**, 189.
6) Motokawa, K., & Ebe, M., Science, 1952, **116**, 92.
7) Yonemura, O. & Nango, R., Nihongankagakkai Zasshi (Jap.), 1953, **57**, 195.
8) Takahashi, R., Fujimaki, N. & Yoshino, T., Iwate Ishi (Jap.), 1955, **7**, 181.
9) Oikawa, T., Tohoku J. Exp. Med., 1953, **58**, 69.

The Tohoku Journal of Experimental Medicine, Vol. 64, Nos. 3-4, 1956

Effect of Blood transfusion and Bleeding on Values of Electric Flicker

By

Kiitiro Suzuki and **Juzaburo Umetsu**
(鈴木 亀一郎) (梅津 重三郎)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, February 24, 1956)

Motokawa and Suzuki[1] showed that a flicker sensation caused by electric stimulation of the eye can be used as a very convenient indicator for the measurement of fatigue. They used rectangular pulses of 20 cycles per second for stimulation. The stimulus voltage was increased from zero at a constant rate of 80 mV per second to determine the threshold S_1 for the appearance of flicker, and then the threshold S_2 for the disappearance was determined by lowering the voltage from a sufficiently high level at the same rate as above. The difference of both sorts of threshold $\Delta S = S_1 - S_2$ was taken as a measure of fatigue. This method of electric flicker has been applied not only to labor hygiene[2] but also to clinical fields.[3] However, physiological factors responsible for changes of electric flicker values have not yet fully been analyzed. Motokawa and Iwama,[4] and Suzuki[5] showed that a remarkable increase of electric flicker values (ΔS) was caused by oxygen lack which was produced by low atmospheric pressure or heavy exercises. In the present experiment we studied this problem in another way. We investigated variations of electric flicker values (EF) in response to bleeding and to transfusion of blood, plasma and saline.

Experimental

Method

For 30 minutes before each operation (blood transfusion, bleeding and plasma injection), subjects were laid down quietly on the bed. A series of measurements was carried out after the rest to obtain control values. Similar measurements were made after the operation at intervals of 10 minutes.

1) We chose 6 healthy subjects for the bleeding. The quantity of bleeding was from 100 to 200 c. c. at a time.

2) We chose 10 subjects for the blood transfusion. They were patients who had been applied laparotomy 3 weeks before. The quantity

of blood transfusion was 100 c. c. at a time.

3) 4 patients were injected 10 c. c. of plasma at one time and 500 c. c. of Ringer solution at another.

The stimulating electrodes were placed at the outer corners of the eyes. The difference between S_1 and S_2 obtained immediately before the operation, was denoted by ΔS_0, and that obtained after the operation by ΔS. The difference, ΔS-ΔS_0, was taken as a measure for the effects of the manipulation. Each ΔS_0 or ΔS represents the mean of ten measurements.

Results

1) Effects of bleeding. In Table I, data concerning bleeding are shown. The quantity of bleeding was 100 c. c. from each of subjects

TABLE I

ΔS-ΔS_0 Values after Bleeding

Subject No.	ΔS_0 in mV	ΔS-ΔS_0 in mV							Quantity of bleeding
	Before bleeding	Immediately after	After bleeding in min. 10	20	30	40	50	60	
14	100	+ 20	+ 60	+ 60	+ 80	− 20	− 40	− 30	100
15	60	+ 20	+120	+190	+200	+320	+210	+120	200
16	60	+ 20	+ 20	+ 40	+ 40	+ 80	+ 40	0	100
17	100	+100	+100	0	− 50	0	0	+ 20	200
18	50	+120	+150	+170	+350	+370			100
19	200	+170	+270	+400	+400	+400	+270	+200	100
Average of 6 cases	96	+ 75	+120	+143	+178	+175	+106	+ 62	

No. 14, 16, 18 and 19, and was 200 c. c. from subjects No. 15 and 17. As can be seen from this table, the value of ΔS-ΔS_0 after the operation increased gradually, and reached a maximum about 30 minutes after the bleeding. A complete recovery was seen in about 60 minutes. The curve A in Fig. 1 was drawn by the average of 6 cases (from No. 14 to No. 19). This figure (curve A) shows a clear maximum at 30 minutes, the average maximal value of ΔS-ΔS_0 being as high as 178 mV. It is without saying that the difference between EFs before and after the operation or ΔS-ΔS_0 is statistically significant.

2) Effects of blood transfusion. The results obtained from subjects No. 1–10 are shown in Table II. As can be seen from this table, the value of ΔS-ΔS_0 for each subject is negative after the operation in contrast

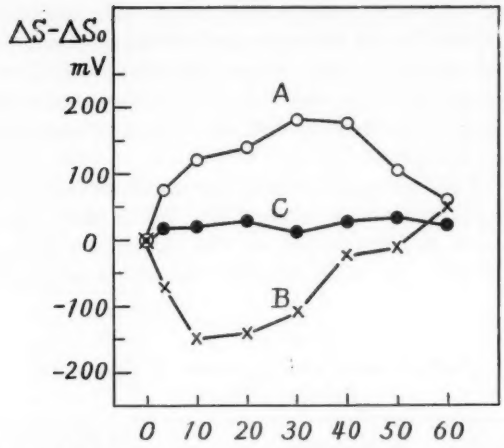


Fig. 1. Changes of electric flicker values after bleeding (A), after blood transfusion (B) and after an injection of blood plasma. Abscissas: time after operations. Ordinates: ΔS-ΔS_0(mV), averages of 6 cases for A, of 10 cases for B and of 4 cases for C.

TABLE II

ΔS-ΔS_0 Values after Blood Transfusion

Subject No.	ΔS_0 in mV	ΔS-ΔS_0 in mV						
	Before transfusion	Immediately after	After transfusion in min. 10	20	30	40	50	60
1	390	− 90	−190	−170	−110	− 30	− 20	+ 10
2	360	−160	−140	− 60	− 60	− 20	0	+ 20
3	300	−100	−100	−120	− 80	− 40	− 40	+ 40
4	380	− 80	−100	−100	− 80	0	+ 40	+ 80
5	600	−170	+220	−140	− 40	0	+ 40	+ 80
6	520	0	− 10	− 60	−100	+ 20	+ 40	+220
7	160	− 60	− 80	− 80	+120	+160	+180	+180
7	680	− 80	−260	−280	−220	− 40	0	− 20
7	360	+ 20	−160	−160	−100	− 80	0	0
8	220	− 20	− 20	−100	−100	− 60	+ 20	+ 80
9	240	− 40	−100	−120	− 80	− 40	0	+ 60
8	240	− 80	−200	−120	−100	0	0	0
10	580	− 60	−300	−320	−300	−220	−120	− 80
Average of 13 cases	386	− 70	−148	−140	−106	− 27	+ 10	+ 51

to the experiment of bleeding mentioned above. The negativity was greatest in most subjects 10–20 minutes after the transfusion. The recovery was completed in 50–60 minutes, as was observed in the case of bleeding. The average values of ΔS-ΔS_0 from 10 subjects are represented by curve B in Fig. 1. It is an interesting fact that bleeding and transfusion have caused opposite changes of EF.

3) Effect of plasma and saline. We measured the value (ΔS-ΔS_0) after an injection of plasma in subjects No. 12, 13 and 1 and after an injection of Ringer's solution in subject No. 8. The results obtained are shown in Table III. In those subjects the values of ΔS-ΔS_0 are found

TABLE III

ΔS-ΔS_0 Values after Injection of Plasma or Ringer's Solution

Subject No.		ΔS_0 in mV Before injection	ΔS-ΔS_0 in mV						
			Immediately after	After injection in min. 10	20	30	40	50	60
12	Plasma 100 c.c.	160	+20	+40	+30	+20	+20	+40	+40
12	Plasma 100 c.c.	30	−10	+10	+10	−10	+10	+10	0
1	Plasma 100 c.c.	600	+40	0	+20	0	+60	+60	+40
8	Ringer's solution 400 c.c.	180		+30	+50	+20	+20	+20	+10
Average of 4 cases		242	+17	+20	+27	+12	+27	+32	+22

generally so low that they seem hardly significant. The average values of all these cases are represented by curve C in Fig. 1. The curve C serves as a control to the experiments of bleeding and blood transfusion. These data indicate that what is responsible for the changes caused by bleeding and by blood transfusion is the decrease and the increase in the number of red blood corpuscles respectively.

Discussion

In this experiment, the injections of the plasma and Ringer's solution have caused no change in EF, but the injection of blood with red blood corpuscles and bleeding of it have caused a conspicuous change of EF, although the directions of the changes are opposite in both cases. In case of the injection of red blood corpuscles, EF decreases, while an increase of EF is caused by deprivation of red blood corpuscles. It may generally be stated that an increase or a decrease of oxygen supply by the blood influences EF.

As our measurements were carried out by means of Motokawa's original method, which is liable to the error due to variations in the skin resistance, one may argue that the changes observed above would have been caused by some variations of skin resistance. Therefore the D. C. resistance of the skin was measured in parallel to the measurements of ΔS in two cases. The curve C in Fig. 2 shows the variations of skin resistance in the two cases in which the effects of bleeding and blood transfusion upon ΔS-ΔS_0 were especially conspicuous. As can be seen in this figure, no

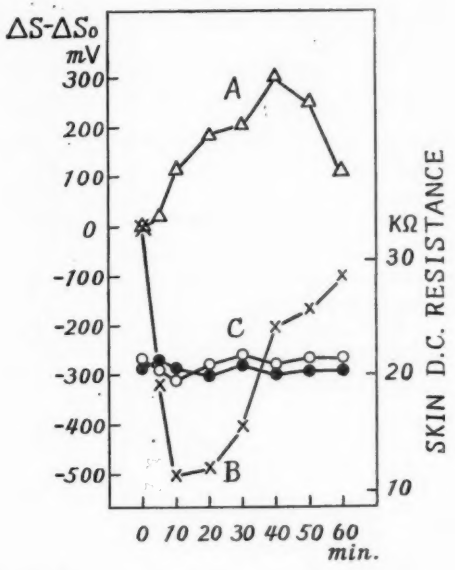


Fig. 2. Variations of skin D. C. resistance (KΩ) in case of bleeding (marked by empty circles) and transfusion (marked by solid circles). Effects upon electric flicker values are also shown : that of bleeding is represented by a curve marked with triangles and that of transfusion by a curve marked with crosses.

significant change took place in the skin resistance in contrast to the considerable effects upon EF. It is obvious from these facts that variations of the oxygen content of the blood are one of the most effective factors responsible for changes of electric flicker values.

Summary

By means of Motokawa's method of electric flicker, we measured for 60 minutes variations of electric flicker values (difference of electric

thresholds for appearance and disappearance of flicker) of 19 subjects after a blood transfusion, a bleeding or an injection of plasma.

1. After the bleeding the value increased gradually, reached a maximum about 30 minutes after the bleeding, and then decreased to the initial value in about 60 minutes.

2. In case of blood transfusion, decreases instead of increases were observed. The value gradually decreased, reached a minimum 10–20 minutes after the transfusion, and then, increased to the initial value in about 60 minutes.

3. In case of the plasma injection, no significant change of electric flicker values could be found.

From these facts it was concluded that an increase of oxygen content of the blood causes a decrease of electric flicker values, and vice versa.

The authors have pleasure in acknowledging their indebtedness to Prof. K. Motokawa who has guided them kindly.

References

1) Motokawa, K. & Suzuki, K., Jap. Med. J., 1942, **1**, 200.
2) Suzuki, K., Tohoku J. Exp. Med., 1950, **52**, 1.
3) Nakamura, T., Saishin Igaku (Jap.), 1953, **8**, 53.
4) Motokawa, K. & Iwama, K., Tohoku J. Exp. Med., 1949, **50**, 319.
5) Suzuki, K., *ibid.*, 1950, **52**, 9.